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Definition and Functional Implications of Gibberellin and Abscisic Acid *cis*-Acting Hormone Response Complexes

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The mechanisms by which *cis*-acting hormone response elements affect transcription is unclear. In this study, we demonstrated that a second "coupling element," identified as O2S, must be present to allow a single copy of either the gibberellin response element (GARE) or the abscisic acid response element (ABRE) to mediate their hormonal effects in the barley Amy32b α-amylase gene promoter. The interactive effects of the O2S and the GARE are constrained positionally and spatially; thus, together they form a gibberellin response complex (GARC). The absolute requirement of the O2S for function of the ABRE demonstrates that these together form an abscisic acid response complex (ABRC). A second copy of the GARE can substitute for the O2S in the GARC, but only in one orientation. By expressing the GARC-containing and ABRC-containing promoters in developing aleurone tissue, we showed that hormonal effects prevent α-amylase gene expression during the second half of grain development, but other mechanisms suppress expression earlier. Our results suggest that the specific sequence serving as a coupling element in a given gene promoter will greatly affect where and when the GARE or ABRE will be able to regulate transcription.

INTRODUCTION

The mechanisms by which plant hormones mediate their effects are unclear. The hormones may be synthesized in different tissues in the same plant; they elicit different effects in different cells, and in some cases the effects of one hormone overlap those of another (Trewavas and Cleland, 1983). In the case of auxin, Gee et al. (1991) showed elegantly that two different classes of auxin-responsive mRNAs had different patterns of expression in different soybean tissues. The fact that most cells expressed one or the other of the mRNAs, yet many cells expressed only one class, led those authors (Gee et al., 1991) to suggest that different types of auxin receptors, or auxin-dependent signaling pathways, might be responsible for increasing transcription from the two different gene classes. To explain how one hormone affects transcription of different genes in different cells is a major goal of those who study hormones and plant development.

No plant hormone receptor has been identified, so an experimental approach toward solving the problem from this direction is not yet possible. An alternative approach would be first to identify in the promoter of a hormonally regulated gene *cis*-acting DNA sequences that were responsible for mediating the effects of that hormone on transcription. Then the strategy would be to determine if those sequences and the factor(s) that interact with them were common to all genes that were transcriptionally regulated by the same hormone. This would provide a basis for working backward to identify the different pathways that might end in a common transcriptional response.

The first step in this approach has been applied successfully to genes whose transcription is increased in response to abscisic acid (ABA) (Marcotte et al., 1989; Mundy et al., 1990; Skriver et al., 1991), and to genes whose transcription is increased by gibberellin (GA) and suppressed by ABA (Skriver et al., 1991; Lanahan et al., 1992). Mundy et al. (1990) identified a highly conserved sequence motif in the promoter of an ABA-regulated rice gene, *rab-16A*, which was footprinted by proteins in nuclear extracts from rice shoot tissue. A similar sequence motif in the promoter of an ABA-regulated wheat gene, Em, was found to interact with a leucine zipper protein (Guiltinan et al., 1990), and mutation of that sequence abolished ABA responsiveness of the promoter when it was transiently expressed in rice suspension culture protoplasts (Guiltinan et al., 1990).

Skriver et al. (1991) demonstrated that the conserved sequence from *rab-16A*, when placed as a tandem repeat of six units upstream from a minimal promoter, mediated increased transcription by ABA in barley aleurone protoplasts. Thus, this sequence, GTACGTGGCGC, was identified as an ABA response element (ABRE). In parallel experiments, Skriver et al. (1991) showed that tandemly repeated copies of GGCCGATAACAAACTCCGGCC could similarly impose increased transcription by GA and its suppression by ABA on the same minimal promoter. Therefore, this sequence was identified as a GA response element (GARE) (Skriver et al., 1991). Huang et al. (1990) provided sequence alignments showing that a motif contained in this GARE, UTAACAUANTCYGG (where U = purine, Y = pyrimidine, and N = any nucleotide), is highly

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conserved in cereal α-amylase gene promoters. It was necessary to link multiple copies of either the ABRE or the GARE to the minimal promoter to observe the proper transcriptional response to the applied hormone (Skriver et al., 1991); yet these sequence motifs were apparently present only as single copies in gene promoters where they participated in transcriptional regulation (Marcotte et al., 1989; Huang et al., 1990; Mundy et al., 1990). This indicated that other *cis*-acting elements in those promoters influenced the ability of the hormone response elements to regulate transcription.

We tested this possibility with a linker scan strategy to dissect the GA-regulated Amy32b barley α-amylase gene promoter in a transient expression system using particle bombardment of intact aleurone layers (Lanahan et al., 1992). These studies showed that there were two separate but physically adjacent elements in the promoter that were essential for GA-induced transcription above a minimal level; mutation or deletion of either one lowered transcription to near baseline. One element, GTAACAGAGTCTGG, was very similar to the sequence defined as a GARE by Skriver et al. (1991) and represented the consensus sequence derived from alignments of Huang et al. (1990); thus, it was reasonable to find that its mutation abolished the effect of GA on transcription. The other element, which we called "O2S," was very similar to a sequence identified as a binding site for the maize endosperm-specific transcriptional regulator, Opaque-2 (Lohmer et al., 1991); this O2S motif formed part of a larger "endosperm box" sequence found in promoters of genes expressed in the starchy endosperm of different cereals (Forde et al., 1985).

Mutation or deletion of three other regions or sequence motifs in the Amy32b promoter, namely the X4–5 interval, the CCTTTT motif, and GCAGTG, which are part of the TATCCATGCAGTG motif, also resulted in decreased expression from the promoter (Lanahan et al., 1992). In none of these instances, however, was the level of expression lowered below 15% of that of the intact promoter. Thus, quantitatively, the O2S and GARE were the most important elements identified. These results showed that other elements participated in establishing the final level of expression from the promoter, but the quantitative data indicated that the O2S and GARE were central to the process. Because mutation of the O2S also essentially abolished GA regulation of transcription, we argued that each element, the GARE and the O2S, required the other to participate in expression from this promoter. We termed the O2S/endosperm box plus the GARE a GA response complex (GARC) and speculated that the requirement for the O2S would functionally limit GA-regulated expression of this promoter to cells in the grain endosperm (Lanahan et al., 1992).

These results led us to ask if other hormone response elements might also require the presence of the O2S to function within the context of this promoter; if so, this would indicate that the requirement for a second element to couple a hormone effect to transcription was not unique to the GARE. The reciprocal question was also relevant: would other functionally defined sequence elements substitute for the O2S? If so, this would support a model where the second "coupling element" could function to determine in which cells and at which times a hormone could affect transcription from a particular promoter. Therefore, we have characterized the spatial requirements that limit the ability of the functionally defined units in the GARC to cooperate in promoting transcription within the context of the Amy32b promoter (Lanahan et al., 1992) and present the results here.

These results demonstrated that the O2S and GARE will only function together in one orientation with respect to each other and with respect to the TATA box. Increasing the distance between these elements drastically decreased transcription from the promoter. Thus, interaction of elements within this GARC is limited by tight positional and spatial constraints. We found that substituting the ABRE for the GARE converted the promoter from one whose transcription was suppressed by ABA to one whose transcription was substantially increased by ABA. As for the GARE, expression of this effect was highly dependent upon the presence of a functional O2S. This result defines an ABA response complex (ABRC). The result demonstrated that a second hormone response element can cooperate with the O2S, and that, in its absence, a single ABRE is unable to function within this promoter context. We also found that substitution of a second GARE for the O2S permitted transcription, which was GA responsive above a baseline level, but only if the two GARE elements were in inverted orientation to each other. This result demonstrated that a different, functionally defined element can substitute for the O2S within a GARC, and thus strengthens our speculation that the nature of the coupling element within a hormone response complex will profoundly affect when and where a transcriptional response to a plant hormone will occur.

RESULTS

Spatial and Positional Constraints on the Function of a GARC

To define the function of each element specifically within the context of the Amy32b promoter, the approach used in our experiments was to change only certain elements, otherwise maintaining the promoter sequence and spatial integrity. Our previous studies (Lanahan et al., 1992) have shown that the presence of an intact O2S and GARE was essential for transcription above a baseline level. Again using the β-glucuronidase (GUS) gene as the reporter for the amylase promoter constructs and ubiquitin promoter/luciferase (Bruce and Quail, 1990) as an internal standard, we further tested the importance of each of these elements by duplicating one or both within the promoter. Results of these experiments are shown in Figure 1. (For the sake of clarity in this and other figures, the positions of the crucial elements are depicted schematically for each promoter construct. The precise sequences of all constructs are described in Methods.)

As previously shown (Lanahan et al., 1992), the −330 truncation of the Amy32b promoter (identified as ML022) gave

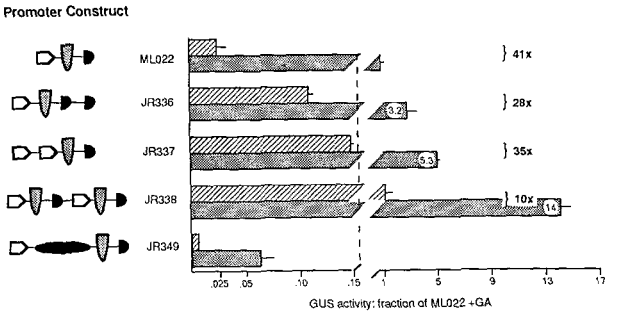

Figure 1. Transcriptional Effects Resulting from Duplicating Portions of the GARC.

Each promoter construct is identified by its number at the left and by a simple drawing to indicate the position and orientation of each of the elements within the GARC. The position of the conserved CCTTTT motif, represented by a vertical stippled cone, is included as a point of reference; the position and orientation of the O2S and the GARE are represented by an open pentagon and a filled semicircle, respectively. The filled oval represents a 70-bp polylinker. Numbers on the abscissa indicate GUS activity expressed as a fraction of that obtained in the presence of GA for the ML022 construct assayed in the same experiment. Numbers to the right indicate the increase in expression, e.g., 41-fold, obtained for each construct in the presence of GA. Numbers within the bars indicate the increase in expression obtained for JR336, JR337, and JR338, respectively, in the presence of GA as compared to ML022. Lines at the end of each bar represent the standard error of the mean for the four samples comprising each data point; diagonally hatched bars indicate no hormone, and the stippled bars indicate 2 × 10^{-6} M GA_3.

high-level expression of the GUS reporter gene, and expression was increased 40-fold in the presence of GA (Figure 1); there are 17 bp between the O2S and GARE in that promoter. When the GARE was duplicated such that 17 bp separated the duplications (JR336), the GA-induced level of transcription was threefold greater than for ML022. When the O2S was duplicated such that 17 bp separated the duplications (JR337), the GA-induced level of transcription was fivefold greater than for ML022. When the entire GARC was duplicated such that 17 bp separated the duplications (JR338), the GA-induced level of transcription was 14-fold greater than for ML022. The increased transcription seen for these constructs was not due to an effect from increasing the distance between the 5′ O2S and 3′ GARE (71 bp in JR338), because when a 70-bp polylinker was inserted between them (JR349), the level of GA-induced transcription decreased to 6% of that obtained for ML022 (Figure 1).

Interestingly, each duplication also increased the level of transcription observed in the absence of hormone such that, for JR338, it was equivalent to the level of transcription obtained in the presence of GA for ML022. An increase in baseline expression in the absence of hormone for constructs that increased the maximum level of expression would be consistent with the fact that the low-pI α-amylase genes have a significant level of expression in the absence of added GA in vivo (Rogers, 1985). One would, however, expect a corresponding increase in the presence of GA. For JR336 and JR337, expression in the presence of GA increased 28- and 35-fold, respectively; these results are not different from those obtained with ML022 in multiple experiments. For JR338, a 10-fold increase in the presence of GA was distinctly lower than would be expected. This is a construct that gave a 14-fold increase over the level of expression obtained with ML022, which itself has a very strong promoter, in aleurone cells that also were transcribing their chromosomal α-amylase genes at high levels. It is possible that some factors essential for transcription of these genes have limited abundance in GA-treated aleurone cells, and such limits prevented higher levels of expression from JR338.

To explain these observations, we hypothesized that it is necessary for protein factors to assemble as a complex on the GARC before transcription can occur. Duplicating the elements that comprise the GARC increases the possibility that stable interaction by factors within the complex will occur; increasing the distance between the elements makes stable interaction less likely between factors that recognize different members of the complex. This hypothesis thus requires either cooperative interactions between factors that recognize both the O2S and GARE or synergistic interactions between factors that recognize those two elements and another factor. (Differences between the two models are discussed below.) Subsequent experiments provide further support for this concept.

We then determined the importance of the orientation of the elements within the GARC relative to each other and to the TATA box; these results are presented in Figure 2. It can be seen that inverting the CCTTTT sequence lowered the level of GA-induced transcription to ~30% of that obtained with

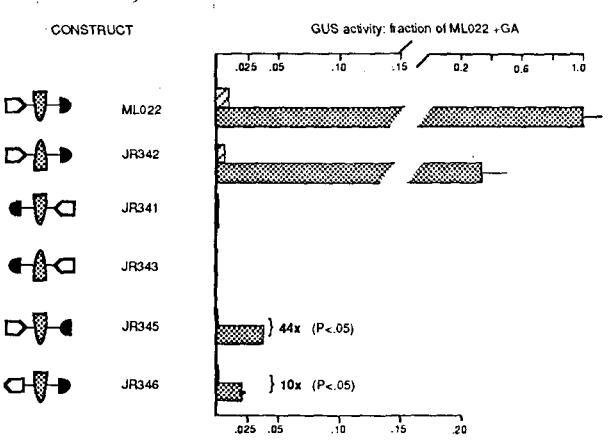

Figure 2. Transcriptional Effects Resulting from Changing the Orientation of Different Elements within the GARC.

Symbols and expression of GUS activity for each construct are the same as given for Figure 1. For JR345 and JR346, 44× and 10× represent 44- and 10-fold increases in transcription obtained in the presence of GA; the probability values are derived by comparison of results obtained from samples incubated in the absence of hormone versus those incubated in the presence of 2 × 10^{-6} M GA_3.

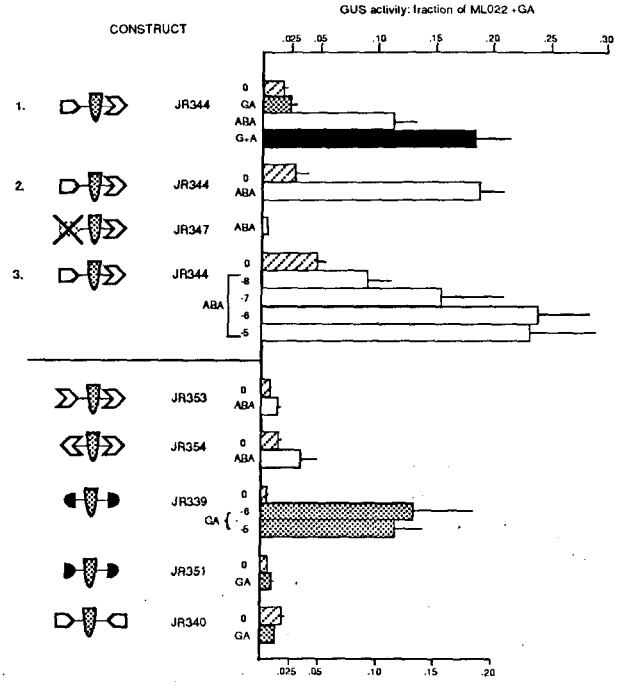

Figure 3. Transcriptional Effects Resulting from Substitution of Different Sequences for the O2S and GARE Elements.

Numbers to the left in the top portion indicate that the results were derived from three separate experiments using JR344. In addition to the symbols described in Figure 1, the arrowhead = ABRE and the pentagon with X superimposed = a mutated, inactive O2S. Symbols representing no hormone (designated 0 to the left of the appropriate bars) and + GA are as given in Figure 1. The open bar indicates the presence of added ABA, and the black bar indicates the presence of 2×10^{-6} M GA_3 plus 2×10^{-5} M ABA. For all experiments except experiment 3, the concentration of ABA was 2×10^{-5} M; for experiment 3, the concentration of added ABA is indicated to the left of the respective bars, where, for example, $-5 = 2 \times 10^{-5}$ M and $-6 = 2 \times 10^{-6}$ M. The concentration of GA_3 used was 2×10^{-6} M, except for JR339, where $-5 = 2 \times 10^{-5}$ M and $-6 = 2 \times 10^{-6}$ M.

ML022; this result is not different from that obtained when the CCTTTT was mutated in the X6 construct (Lanahan et al., 1992). When the orientation of the entire GARC was inverted (JR341 and JR343), little transcription above background was obtained; these results demonstrated that the GARC has an absolute requirement for orientation relative to the transcription initiation site. The orientation of the elements within the GARC was similarly very important. When either the GARE or the O2S was inverted (JR345 and JR346, respectively), transcription was still induced by GA but the levels declined to 4% (JR345) and 2% (JR346) of that obtained with ML022. These results are consistent with our hypothesis that specific interactions must occur between protein factors that recognize different elements within the GARC before transcription can be initiated; by inverting one element, we presumably made it more difficult for a stable complex to form. These results also emphasize that the concept of a defined complex composed of the O2S and GARE is legitimate: clearly, it is not similar to what would be expected if either acted simply as an independent transcriptional enhancer, where the placement of each element within the promoter would be relatively unimportant.

Functional Definition of an ABRC

We then wanted to determine if other functionally defined sequence elements could substitute for either the GARE or the O2S. Our first approach was to determine if other elements could function properly in place of the GARE. Results from these experiments are presented in the upper part of Figure 3; numbers on the left side indicate that these results were from three separate experiments.

It can be seen that a construct, JR344, where the ABRE replaced the GARE, consistently gave a fivefold to eightfold increase in transcription in the presence of 10^{-5} M ABA; this was a high level of expression, being equivalent to ~20% of that obtained with the strong ML022 promoter in the presence of GA (Figure 3, experiments 1 to 3). The presence of 10^{-6} M GA alone or in combination with ABA did not affect transcription from this promoter ($P > 0.1$ ABA versus GA + ABA; experiment 1). The presence of an intact O2S was absolutely required to observe this effect; when it was mutated (JR347), transcription diminished to a very low level (experiment 2). It is important to note that the endogenous GUS activity from control aleurone layers was ≤10% of the value for JR347 (data not shown), so that even this low level of transcriptional activity was measurable. To allow relative comparisons in subsequent experiments, we will refer to this level of ~1% of that obtained with ML022 in GA-treated tissue as "baseline."

The presence of the ABRE was also absolutely required to observe this ABA effect; expression of the X11 construct, which mutates the GARE (Lanahan et al., 1992), was at baseline level in the presence of ABA (data not presented). The effect of ABA on transcription was dose dependent, with a maximum observed at 10^{-6} M (experiment 3). Therefore, we concluded that, in the presence of an intact O2S, the single ABRE functions in a manner identical to that previously described in the aleurone protoplast system (Skriver et al., 1991). These results defined the O2S–ABRE unit as an ABRC.

Only Certain Sequences Cooperate with a Hormone Response Element to Form a Functional Complex

Our second approach was to determine if other sequences could substitute for the O2S in this system. If so, it would support a model where the type of second "coupling element" might function to determine where and when a hormone response element would be able to influence transcription. This approach is complicated by the fact that only sequence elements that are known to be functional in aleurone cells would provide an

adequate test; for that reason, we limited our attention to the two hormone response elements.

Results obtained when an ABRE was substituted for the O2S in JR344 (Figure 3) emphasized the specificity of the system. Regardless of whether the second ABRE was in tandem (JR353) or inverted (JR354) orientation, transcription was only slightly above baseline and the presence of ABA had little or no effect; thus, a functional complex requires more than just the presence of two elements in close proximity. These results also demonstrated that the low level of expression obtained with JR347, with a mutated O2S, did not result from the inadvertent introduction of a transcriptional repressor.

Substantially different results were obtained, however, when a GARE was substituted for the O2S. The presence of a GARE in inverted orientation (Figure 3, JR339) gave a 32-fold induction of transcription by GA to a level that was ~10% of that obtained with ML022. We do not know if this effect was due to the GARE motif itself or to new sequence motifs created at the junctions, but, nevertheless, the result demonstrates that a sequence apparently much different from the O2S can partially substitute for its function. This effect of the second GARE in place of the O2S was absolutely dependent on its orientation; when the two GARE elements were in tandem orientation (Figure 3, JR351), transcription was essentially at baseline level and was not affected by the presence of GA. A construct in which two O2S elements were present in inverted orientation (Figure 3, JR340) also gave a level of transcription that was only slightly above baseline; this further emphasizes the specificity of the results obtained with JR339. We concluded that only certain sequences are able to substitute for the O2S within either a GARC or an ABRC.

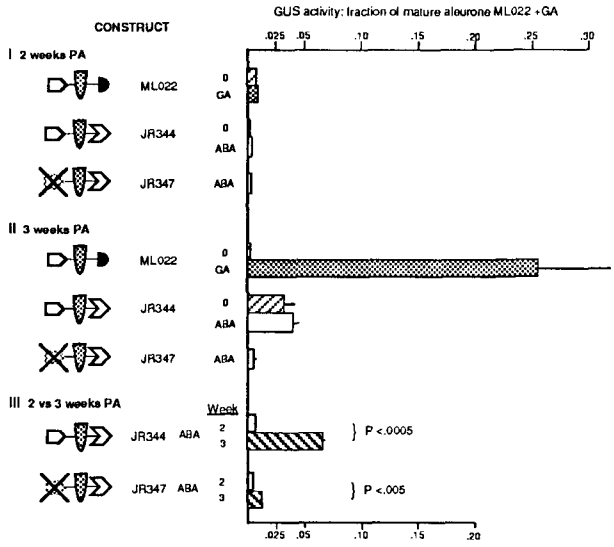

Figure 4. Activity of GARC- and ABRC-Containing Constructs in Developing Barley Grains.

Symbols and designations for hormones are as given in Figures 1 and 3. Two weeks postanthesis (PA) indicates grains that were removed from the plant between 2 and 2.5 weeks PA; 3 weeks PA indicates grains removed between 3 and 4 weeks. Three separate experiments are shown in this figure, indicated as I, II, and III. In experiment III, all samples were incubated in the presence of 2×10^{-5} M ABA; the open box indicates grains 2 weeks PA and the diagonally hatched box indicates grains 3 weeks PA. The probability values to the right derive from comparison of results obtained from the samples 2 and 3 weeks PA for each construct.

The ABRC and GARC Used to Probe a Developmental Switch in Gene Expression during Grain Development

We have shown above (Figure 3, top) that the Amy32b GARC can be converted to an ABRC by simply switching the respective hormone response elements. This means that all other *cis*-acting DNA sequences known to affect expression of that promoter (Lanahan et al., 1992) are undisturbed and should function normally; presumably, this explains why JR344 (with the ABRC) was expressed at high levels in the presence of ABA. The availability of two otherwise identical promoters that differ only in a single hormone response element (ML022 versus JR344) allows us to test whether it is simply the hormonal environment that prevents expression of α-amylase during grain development (Cornford et al., 1986).

We therefore analyzed expression of the two different types of promoters in aleurone layers from grains at different stages of development. Under growth conditions in our greenhouse, grains are mature at ~6 weeks postanthesis. At 2 weeks postanthesis, the grains reach mature size, but the aleurone cells lack visible pigmentation and the embryos are easily dislodged from the endosperm with even minimal manipulations. At 3 weeks postanthesis, approximately half of the grains will have aleurone layers with some blue pigmentation, and the embryos will firmly adhere to the endosperm. As shown in Figure 4, experiment I, aleurone layers from grains 2 weeks postanthesis did not express either ML022 or JR344 at levels above baseline (defined by expression obtained with JR347). In contrast, both ML022 and JR344 were expressed at levels significantly above background in grains that were 3 weeks postanthesis (experiment II). These results were reproducible in multiple experiments; they were not due simply to an inability of grains 2 weeks postanthesis to express genes on foreign DNA because the magnitude of luciferase expression obtained from the ubiquitin promoter–luciferase internal standard was indistinguishable in aleurone layers 2 and 3 weeks postanthesis (data not presented).

The construct with the ABRC, JR344, was expressed at similar levels regardless of the addition of exogenous ABA; this most likely reflects the presence of micromolar endogenous levels of that hormone (Jacobsen and Chandler, 1987) in grains 3 weeks postanthesis. In this regard, the ability of ML022 to be expressed at high levels in the same tissue was somewhat surprising; this probably reflects the fact that ABA levels would be expected to decrease during the 40-hr incubation (Cornford et al., 1986) following particle bombardment. In other

experiments (data not shown), we found that the expression of this construct in grains 3 weeks postanthesis was decreased 75% in the presence of 1 μM GA plus 10 μM ABA as compared to expression in 1 μM GA alone. The results presented in Figure 4, experiments I and II, were obtained with grains from the same plants, assayed more than 1 week apart. To exclude the possibility of differences imposed by assays done on different days, grains at the two stages of development from different plants were assayed in the same experiment for their ability to express JR344 and JR347; these results are presented in Figure 4, experiment III. First, it can be seen again that the level of expression of JR344 and JR347 were the same in aleurone 2 weeks postanthesis. As predicted from the prior experiments, JR344 was transcribed at significantly higher levels in aleurone 3 weeks postanthesis. Interestingly, expression of JR347 was also significantly higher in aleurone 3 weeks postanthesis, albeit still at a low level.

These results together demonstrate that the low level of transcription observed in aleurone cells 2 weeks postanthesis was not affected by the type of hormone response element present within the promoter. Increased transcription in aleurone cells 3 weeks postanthesis was appropriate for the type of hormone response element present, indicating that hormone-mediated effects on transcription were important in regulating the promoters in those cells. The result with JR347 in Figure 4, experiment III, also indicates that factors acting outside of the ABRC contributed to the increased expression of these constructs in more mature grains. At this point in our studies, we cannot identify one single element that might explain the low level of expression in cells 2 weeks postanthesis, but the profound suppression of transcription in those cells is similar to that observed with the JR347 mutation. We hypothesized that the O2S may play a key role in suppressing transcription early in grain maturation, but this speculation can only be tested after we identify other sequence elements that efficiently substitute for the O2S in the ABRC and then use them in similar experiments.

DISCUSSION

Our goals are to understand the requirements for interaction of a plant hormone response element with other regulatory *cis*-acting DNA sequences and to use that knowledge to study the molecular mechanisms by which the hormone affects gene expression during plant development. The first direct indication that a plant hormone response element requires a second element to couple hormone effects to the transcriptional apparatus came from work by Skriver et al. (1991). These investigators showed that six tandemly repeated copies of either the ABRE or the GARE, but not a single copy, when fused to a minimal promoter could confer proper hormonal regulation of transcription upon that promoter. These ABRE and GARE sequences, however, are only single-copy elements in promoters where they have been identified (Marcotte et al., 1989; Huang et al., 1990; Mundy et al., 1990).

Our initial studies of the interaction of the GARE with the O2S sequence in the Amy32b promoter (Lanahan et al., 1992) provided an explanation for this apparent paradox; these results indicated that the O2S sequence had to be present before the function of the GARE could be expressed in promoting transcription, and we termed the association of the O2S–GARE a GARC. This concept implied that it was that specific association that permitted function of the single-copy GARE to be seen. Because several other sequences in that promoter also had substantial effects upon the level of GA-induced transcription (Lanahan et al., 1992), it was possible that perhaps the physical association of the O2S and GARE was only coincidence.

The results presented here now establish that the GARC is a functionally defined complex. Transcription above only a few percent of that obtained with the intact promoter requires that the O2S and GARE be oriented exactly in one direction with respect to each other. The two elements must be positioned close to each other; if that distance is increased by 70 bp, transcription drops to a few percent of that for the intact promoter. We have not, however, defined the exact limits on this spacing, and, as noted before (Lanahan et al., 1992), this varies among different amylase promoters. In addition, the complex can only be oriented in one direction with respect to the TATA box; thus, the complex does not function as one large orientation-independent enhancer. These results in aggregate, coupled with the finding that duplicating the GARC increased the level of expression from this already strong promoter by 10-fold, indicate that formation of a complex of transcription factors over the GARC is likely to be a crucial initial step in promoting transcription.

Results from analyses of protein factors that interact with these sequences are consistent with our findings. Lanahan et al. (1992) summarized DNase I protection experiments where the GARE and O2S elements were shown to interact with proteins from barley aleurone cell nuclear extracts. Similar experiments have been reported by Rushton et al. (1992), who studied the interaction of nuclear proteins from oat aleurone protoplasts with a low-pI wheat α-amylase gene promoter closely related to our Amy32b promoter. These authors identified specific protein interactions with what we have described as the GARE and O2S elements. In a different approach, Sutliff et al. (1992) used gel retardation assays to demonstrate the presence of a GA-induced factor that interacted with an oligonucleotide carrying the GARE motif. So far there are no published reports of studies where interactions between protein factors that recognize different sequence motifs have been identified.

Two general types of models could be postulated from our analyses of *cis*-acting elements. First, it is possible that an O2S–GARE combination of elements is required because the factor(s) that interacts with the GARE is unable to form a stable association with DNA alone. The factor(s) that interacts with the O2S would physically associate with the GARE binding

factor, thereby allowing this complex to be stably maintained on the DNA. This first "cooperative" model is distinguished from the second "synergistic" model. In the synergistic model, factors that independently bound to the GARE and to the O2S would together interact with a third factor that is required for transcription above a baseline level. Although this third factor could interact with either of the other factors alone, formation of a stable complex would be greatly enhanced by the presence of both the GARE and O2S binding factors together. Viviparous-1 is a plant transcription factor that presumably must interact with other proteins on DNA because it has no apparent DNA binding specificity of its own (McCarty et al., 1991); this could provide an example of how such a synergistic system might exist.

Results obtained with the ABRE and with substitution of a second GARE for the O2S are consistent with both models. When an ABRE was substituted for the GARE, transcription from the promoter was increased eightfold by ABA and was insensitive to the presence of GA. This effect, however, was absolutely dependent upon the presence of the O2S. Mutation of the O2S or substitution of a second ABRE for the O2S lowered transcription to baseline. These results demonstrated that the ABRE also requires a second element before its effects can be imposed upon transcription. These findings may help to explain why six tandemly repeated units of either the ABRE or the GARE were sufficient to impose proper hormonal control on a minimal promoter in the aleurone protoplast transient expression system (Skriver et al., 1991). Presumably, the availability of multiple binding sites for the hormone response element binding factor(s) presented either the opportunity for cooperative interactions that would stabilize the binding factor on the DNA or for multiple interactions with a putative third factor that would allow the binding factor to be maintained in the transcription complex. We have not tested the effect of more than two hormone response elements in any one of our promoter constructs because the goal of our studies was to understand the function of a GARE or an ABRE within the spatial and positional constraints imposed by other functional elements in the promoter (Lanahan et al., 1992).

We used the availability of two promoters that differed only with respect to whether they contained a GARC or an ABRC to investigate the role of those two hormones in suppressing expression of α-amylase genes during grain development. It is well established that aleurone cells are not normally able to express genes for α-amylase and other hydrolytic enzymes until the grain matures (Black et al., 1983; Cornford et al., 1986). After a certain stage, approximately midway during grain development, expression of these genes can be induced in the presence of GA by first allowing the grain to dry (Black et al., 1983; Cornford et al., 1986). Neither the reason for the inability to respond to GA early in development nor the reason why drying can induce this ability later in development is understood. One possible explanation (Cornford et al., 1986) would involve the relatively high levels of ABA present in development (Jacobsen and Chandler, 1987; Quatrano, 1987), because micromolar levels of that hormone can prevent GA-induced expression of α-amylase genes in mature aleurone cells (reviewed in Jacobsen and Chandler, 1987; Skriver et al., 1991).

We found that prior to 3 weeks postanthesis, a time approximately midway in grain development, neither promoter was transcribed above a baseline level; after that time, both promoters were expressed and responded appropriately to the hormonal environment. Three weeks postanthesis corresponds to the time at which mitoses cease in developing aleurone, the end of the "differentiation stage" of endosperm development (Bosnes et al., 1992). It would not be surprising to find that a change in expression of certain genes accompanied this alteration of growth potential in aleurone tissue. Our results indicated that the presence of significant levels of ABA and the absence of GA during the latter half of grain development are major determinants in suppressing α-amylase gene transcription. They also demonstrated that another, yet undefined, mechanism is responsible for the genes' silence earlier in development.

When a second GARE was substituted for the O2S, GA-regulated transcription was measured at a level ~10% of that obtained with the intact promoter, but this also was absolutely dependent upon the orientation of the second GARE. This result demonstrated that other sequences can substitute for the O2S. Therefore, we identified the O2S or another sequence that substitutes for it as a "coupling element," because it couples the hormone response to transcription. This term would not distinguish between a cooperative versus synergistic model for the action of such a coupling element. Our results suggest that many different sequences may be able to perform this coupling function. We speculate that the nature of the coupling element will profoundly affect the cell type and temporal pattern of gene expression induced by GA or ABA, because factors binding to the coupling element may be regulated in different ways. This concept is substantially different from that posed, for example, by the simple animal steroid hormone receptor–response element interaction (Fuller, 1991; Miner and Yamamoto, 1991). It is much more consistent with data regarding interactions of the glucocorticoid receptor with other transcription factors on recently described composite response elements (Fuller, 1991; Miner and Yamamoto, 1991). The general applicability of this concept will be tested when response elements for other phytohormones, such as ethylene and auxin, are defined. The validity of the concept and the mechanisms involved will only be tested definitively when an in vitro transcription system using purified factors is available for one of the hormonally regulated gene systems.

METHODS

General Methods

The methods for preparing de-embryonated half grains of Himalaya barley, introducing construct DNA by particle bombardment, preparing extracts from bombarded tissue, and assaying luciferase and β-glucuronidase (GUS) have been described previously (Lanahan et al., 1992).

Construct		Sequence (O2S / GARE)	
X10	(-151)	TTGAC TTGACCATCATCTAGACACCTTTTCTCGTAACAGAGTCTGG	(-105)
JR339		TTGACcagactctgttacTCTAGACACCTTTTCTCGTAACAGAGTCTGG	
JR340		TTGAC TTGACCATCATCTAGACACCTTTTCTCGatgatggtcaaGG	
JR341		TTGACcagactctgttacTCTAGACACCTTTTCTCGatgatggtcaaGG	
JR342		TTGAC TTGACCATCATCTAGACAgaaaaggTCGTAACAGAGTCTGG	
JR343		TTGACcagactctgttacTCTAGACAgaaaaggTCGatgatggtcaaGG	
JR344		TTGAC TTGACCATCATCTAGACACCTTTTCTCGTAcgtggcgc	
JR345		TTGAC TTGACCATCATCTAGACACCTTTTCTCGccagactctgtta	
JR346		TTGA gatgatggtcaagTCTAGACACCTTTTCTCGTAACAGAGTCTGG	
JR347		TTGAC gctagcTCTAGACACCTTTTCTCGTAcgtggcgc	
JR349		TTGAC TTGACCATCATCTAGcctagactgcagtcgatgcatactagtt tgctagctctagactgcagtcgatgcatactagttgactagACACCTTTTCTCGTAACAGAGTCTGG	
JR351		TTGA taacagagtctggTCTAGACACCTTTTCTCGTAACAGAGTCTGG	
JR352		TTGAC TTGACCATCATCTAGACACCTTTTCTCGcttgaccatcatG	
JR353		TTGA gtacgtggcgcTCTAGACACCTTTTCTCGTAcgtggcgc	
JR354		TTGA gcgccacgtATTCTAGACACCTTTTCTCGTAcgtggcgc	

Figure 5. Sequences for New Constructs.

The numbers of the constructs are listed to the left, and the sequence of each within the region altered by mutations is presented to the right (in 5′ to 3′ orientation). The sequence of the X10 mutation (Lanahan et al., 1992) is presented at the top to provide a point of reference. The sequence represents nucleotides between positions −151 and −105 of that promoter; the TATA box is located at −30. The position of the O2S is enclosed in a box and that of the GARE is enclosed in an oval. The position of the XbaI mutation in X10 (Lanahan et al., 1992) is underlined. Mutated nucleotides in the new constructs are written in lowercase letters. For JR349, the long polylinker insertion continues into the second line of the construct sequence.

The GUS assays in this study included 20% methanol to minimize endogenous activity (Kosugi et al., 1990). As in that study, each experiment introduced the test construct containing the GUS marker along with a ubiquitin promoter–luciferase reporter internal standard construct (Bruce and Quail, 1990) in a 2:1 molar ratio. All GUS assay values were corrected for transfection efficiency using the luciferase values as described previously (Lanahan et al., 1992). Each data point presented in Figures 1, 2, 3, and 4 came from four replicate samples. Statistical comparisons between results within a given experiment were made using Student's *t* test. To permit comparisons of results between different experiments, GUS activity values from test constructs were expressed in Figures 1, 2, 3, and 4 as fractions of the values obtained from the intact promoter construct, ML022, which was assayed in mature aleurone layers in each experiment.

DNA Constructions

Constructs X5, X6, X7, and X10 have been described previously (Lanahan et al., 1992). JR336 was constructed by inserting the HindIII-XbaI fragment from X7 into the same interval of X6, JR337 by inserting the HindIII-XbaI fragment from X6 into the same interval of X5, and JR338 by inserting the HindIII-XbaI fragment from X7 into the same interval of X5. For the other constructs used in this study, the base upon which alterations were made was X10. As shown in Figure 5, this mutation changed six nucleotides to insert an XbaI site between the O2S and CCTTTT, a mutation that did not affect hormone response or level of expression (Lanahan et al., 1992).

Each alteration of the various elements, O2S, GARE, or CCTTTT, was accomplished by constructing an oligonucleotide (purchased from commercial sources) with the desired sequence that overlapped the X10 XbaI site and the sequence to be changed and extended at least 12 nucleotides into flanking sequences. This oligonucleotide was used in polymerase chain reaction (PCR) incubations, where the second oligonucleotide was either a reverse pUC/M13 sequencing primer (AACAGCTATGACCATG, New England Biolabs, Beverly, MA) or an oligonucleotide primer (GGGATCCTCTCTTGCTGTGCT) that overlapped the unique BamHI site positioned in front of the ATG translation initiation codon in X10.

PCR protocols were as described by Holwerda et al. (1992). PCR products were precipitated by adding an equal volume of 2.5 M NaCl and 20% polyethylene glycol, incubating on ice for 1 hr, then pelleting at room temperature for 10 min at 10,000*g*. The pellets were dissolved in standard restriction enzyme buffer and digested with either HindIII and XbaI or XbaI and BamHI, gel purified, and inserted into the appropriate interval of X10 or a subsequent derivative. All resultant constructs were sequenced across the interval derived from PCR to ensure that the appropriate mutation was present and that no additional mutations had been introduced by the PCR process itself. The sequences of the resultant constructs are presented in Figure 5.

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