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A wheat α-*Amy2* promoter is regulated by gibberellin in transformed oat aleurone protoplasts

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Gibberellin (GA_3)-responsive aleurone protoplasts isolated from *Avena sativa* have been successfully used as a transient expression system to analyse promoter fusions between the wheat α-amylase gene α-*Amy2/54* and the reporter gene *GUS*. Following PEG-mediated uptake of plasmid DNA, transient expression directed by the α-*Amy2/54* promoter was found to be regulated in the same way as the endogenous oat α-amylase genes. Expression was thus dependent on the inclusion of GA_3 in the protoplast incubation media, could not be detected before a lag phase of 2 days following transformation and was inhibited by simultaneous addition of abscisic acid (ABA) with GA_3 to the media. In contrast, expression from the CaMV 35S promoter in the same system was not affected by GA_3 or ABA and could be detected 1 day after transformation. Introduction of a further three different promoters into the aleurone protoplasts confirmed that GA_3 specifically controlled transient expression from the α-*Amy2/54* promoter only. Promoter deletions of the α-*Amy2/54:GUS* fusion demonstrated that sequences within 300 bp of the start of transcription of the gene were sufficient to direct high-level expression that was regulated by GA_3 and ABA.

Key words: GA_3-regulated/transient expression/oat aleurone protoplasts/α-amylase/β-glucuronidase

Introduction

The aleurone cells of cereal grains provide a unique opportunity to study developmental and hormonal regulation of plant gene expression (see Akazawa and Miyata, 1982). Firstly, the aleurone layer, which surrounds the starchy endosperm, is comprised of a single cell type that in the mature grain may be dissected free of any other living tissue by the simple removal of the embryo and scutellum. Secondly, the major activity of the aleurone layer is the synthesis of hydrolytic enzymes and their secretion into the endosperm during germination and this process is induced by the plant hormone gibberellin (GA_3). Thirdly, it has been demonstrated that production of several of the enzymes results from increased levels of mRNA (Lazarus *et al.*, 1985; Rogers *et al.*, 1985; Mundy and Fincher, 1986; Baulcombe *et al.*, 1987a), and for one enzyme at least, α-amylase, this is due to an increase in the rate of transcription of its genes (Jacobsen and Beach, 1985; Zwar and Hooley, 1986).

α-Amylase, a starch endo-hydrolase, exists in many isoforms that can be separated on isoelectric focusing gels (Sargent, 1980; Jacobsen and Higgins, 1982). Previous work showed that the isozymes present in germinating grain of wheat were the products of two distinct multigene families residing on the group 6 (α-*Amy1*) or group 7 (α-*Amy2*) chromosomes (Lazarus *et al.*, 1985). Expression of both gene families is dependent on the presence of GA_3, but α-*Amy1* isozymes constitute the more abundant group and can be detected in advance of the α-*Amy2* isozymes during germination (Sargent, 1980). Accumulation of the mRNA for both groups commences after 12–24 h, but that specific for the α-*Amy1* group peaks and declines after 48 h, while that from the α-*Amy2* group continues to increase (Lazarus *et al.*, 1985). A subset of α-*Amy2* genes is also expressed in developing grain where a third α-amylase gene family (α-*Amy3*) is expressed (Baulcombe *et al.*, 1987b; Huttly *et al.*, 1988). In the latter tissue, expression of the α-amylase genes is not thought to be GA_3 dependent.

A preliminary search for sequence motifs important for control of expression in the upstream sequences of five wheat α-*Amy2* genes showed that there were regions of close sequence similarity between four of the genes compared up to 300 bp upstream of the start of transcription. In addition two barley α-*Amy2* type genes contained the same sequences (Knox *et al.*, 1987; Whittier *et al.*, 1987; Huttly *et al.*, 1988). Extensive regions (>600 bp) of similarity may also be found in the aligned upstream regions of different α-*Amy1* genes (R.A.Martienssen and D.C.Baulcombe, submitted), but comparisons between α-*Amy1* and α-*Amy2* genes indicated that apart from some similarity around potential TATA box motifs there was no obvious homology. Hence, in order to determine the location of sequences that might mediate, for example, the common response to GA_3, a functional assay system was required. In the absence of a method of producing transgenic wheat plants, promoters from some isolated wheat genes have been stably transferred to tobacco where they retain their correct developmental and environmental regulation (Lamppa *et al.*, 1985; Colot *et al.*, 1987). There is, however, no tobacco tissue analogous to the aleurone layer, and so analysis of a wheat α-amylase construct in this plant seemed inappropriate. We therefore investigated the use of protoplasts isolated from aleurone layers as a transient expression system for the analysis of a wheat α-*Amy2* promoter (α-*Amy2/54*; Huttly *et al.*, 1988). Isolated wild oat (*Avena fatua*) and barley aleurone protoplasts retain their differentiated state, responding in many ways as intact aleurone cells, as seen by their ability to produce hydrolytic enzymes, including α-amylase, in response to the application of GA_3 and an inability to re-form cell walls and divide (Hooley, 1982; Jacobsen and Beach, 1985). The natural target cell for analysis of a

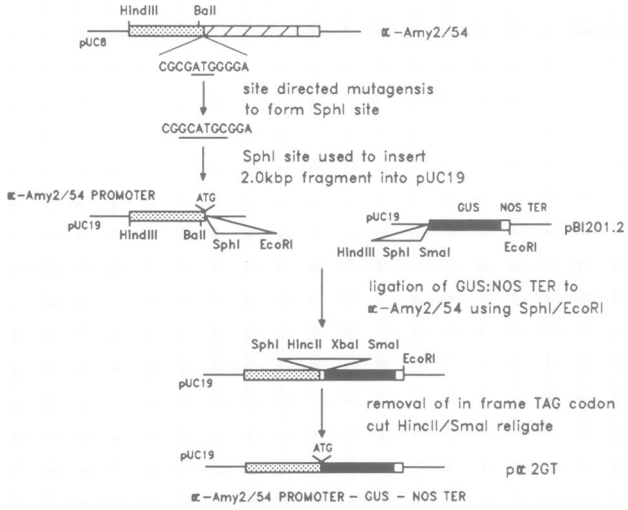

Fig. 1. Diagram showing the construction of pα2GT, which contains upstream sequences from α-*Amy2/54* fused translationally to the *GUS* gene and *NOS* polyadenylation signal (*NOS TER*) in pUC19. This was achieved by introduction of an *Sph*I site into the α-*Amy2/54* sequence at the initiating methionine codon. The introduced site was used to insert a 2.0 kbp fragment of promoter sequence into pUC19. The *GUS* gene and *NOS TER* were inserted into the construct using the common restriction sites *Sph*I and *Eco*RI. Removal of an in-frame termination codon, contained in the polylinker sequence of the fusion, produced pα2GT, which codes for a fusion protein of β-glucuronidase containing a 10 amino acid extension including the initiating methionine codon of α-*Amy2/54*.

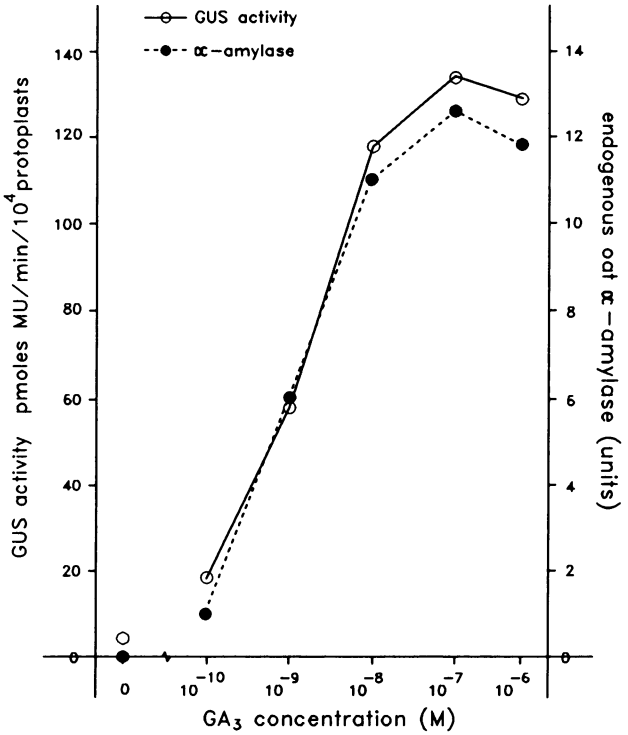

Fig. 2. GA_3-regulated expression from pα2GT. Oat aleurone protoplasts were transformed with 20 μg of the plasmid pα2GT and divided equally into six. The six aliquots of protoplasts were incubated in media containing increasing concentrations of GA_3, as indicated. Production of β-glucuronidase by the protoplasts was assayed after 5 days and expressed as pmol MU/min/10^4 protoplasts. Endogenous oat α-amylase secreted into the incubation media by the transformed protoplasts after 5 days incubation was determined using alphachrome substrate and expressed relative to units of barley malt α-amylase (Sigma).

Table I. Effects of GA_3 and ABA on the transient expression of *GUS* directed by CaMV:GUS and pα2GT

Construct	β-Glucuronidase activity[a]			Oat α-amylase[b]		
	$-GA_3$	$+GA_3$	$+GA_3$ +ABA	$-GA_3$	$+GA_3$	$+GA_3$ +ABA
CaMV:GUS	1146	1156	1100	0	2.7	0
pα2GT	2.3	140	2.0	0	2.3	0

Protoplasts following transformation were divided into three and incubated with no GA_3, 10^{-6} M GA_3 or 10^{-6} M GA_3 + 2.5×10^{-5} M ABA for 5 days before analysis. Production of endogenous oat α-amylase by the transformed protoplasts is also indicated.

[a]pmol MU/min/10^4 protoplasts.

[b]Units relative to barley malt α-amylase.

wheat gene would have been protoplasts isolated from wheat aleurone layers; unfortunately we were unable to produce wheat protoplasts in sufficiently high yields to be transformed. We thus focused our attention on the use of oat aleurone protoplasts.

This paper shows that transformed cultivated oat (*A.sativa*) aleurone protoplasts retained the ability to respond to GA_3 and that transient expression from a construct that contained a promoter fragment from α-*Amy2/54* fused at the start of translation to the reporter gene β-glucuronidase (*GUS*) was specifically controlled by GA_3 in a manner similar to the expression of the endogenous oat α-amylase genes. In addition, deletion analysis of the α-*Amy2/54* promoter delineated the 5′ end of a region necessary for high-level GA_3-dependent expression to within 300 bp from the start of transcription.

Results

Transformation of oat aleurone protoplasts

To optimize the transformation procedure and the detection of β-glucuronidase (GUS) activity, the plasmid CaMV:GUS containing the CaMV 35S promoter to drive expression of *GUS* was used. Highest levels of expression were obtained when the protoplasts were transformed according to the procedure outlined in Materials and methods. It is not known what proportion of protoplasts were transformed by this method. Detection of the GUS activity produced by transformed protoplasts was initially difficult because during culture aleurone protoplasts were found to produce polyphenols that quenched the fluorescence from MU (the fluorogenic product of the GUS assay). This problem was overcome by first washing the protoplasts free of incubation medium and then crude purification of a protoplast extract by addition of Polyclar to the GUS buffer and passage of the extract through a Sephadex G25 column. Reliable measurements of GUS activity were then obtainable from samples containing as few as 0.15×10^4 viable protoplasts transformed with CaMV:GUS. Isolation of large numbers of cultivated oat aleurone protoplasts ($>1-2 \times 10^6$) proved impracticable, although with survival rates of between 5 and 20%, transformation of $<10^5$ protoplasts was possible. Nevertheless, it was not normally possible to analyse more than six promoter constructs at one time. The results presented in this paper are therefore representative experiments that were repeated several times. Qualitatively similar data were obtained each time but absolute levels of

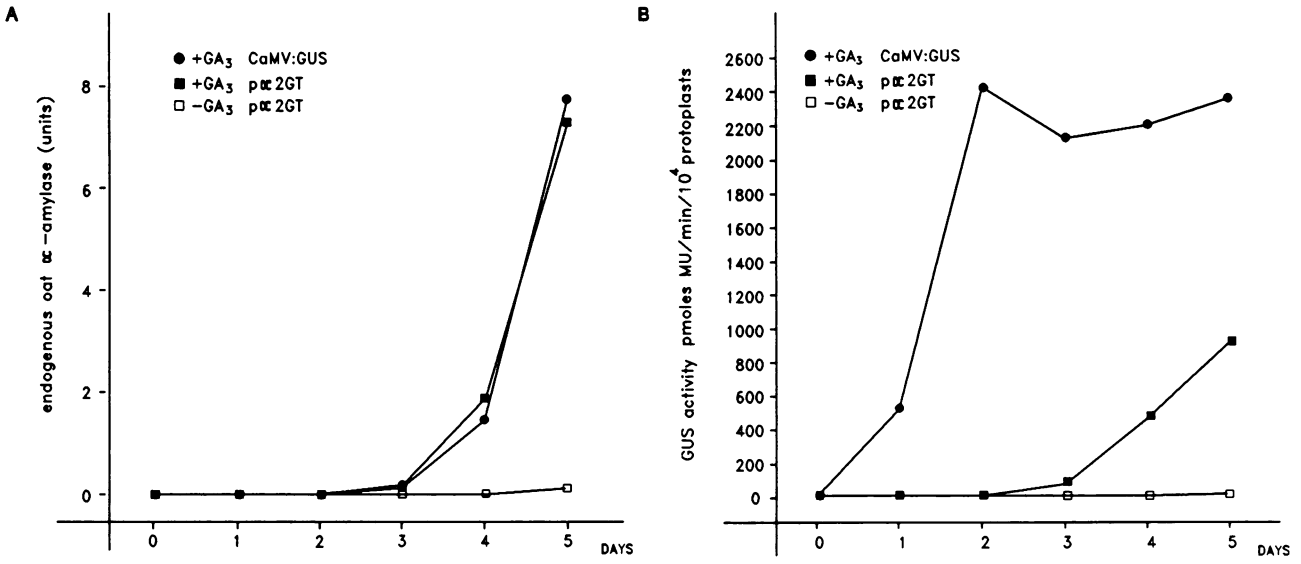

Fig. 3. Time course of expression of endogenous oat α-amylase and β-glucuronidase activity by aleurone protoplasts transformed by pα2GT or CaMV:GUS. (**A**) Production of endogenous oat α-amylase by transformed aleurone protoplasts over 5 days of incubation in the presence or absence of 10^{-6} M GA_3. α-Amylase activity is expressed relative to units of barley malt α-amylase. (**B**) Production of GUS by transformed protoplasts over 1−5 days incubation. Aleurone protoplasts were transformed with either 20 μg of CaMV:GUS or pα2GT. Protoplasts transformed with pα2GT were divided into two and incubated in the presence and absence of 10^{-6} M GA_3. Protoplasts transformed with CaMV:GUS were incubated in the presence of 10^{-6} M GA_3. GUS activity produced by transformed protoplasts, expressed as pmol MU/min/10^4 protoplasts, is plotted over the 5-day period.

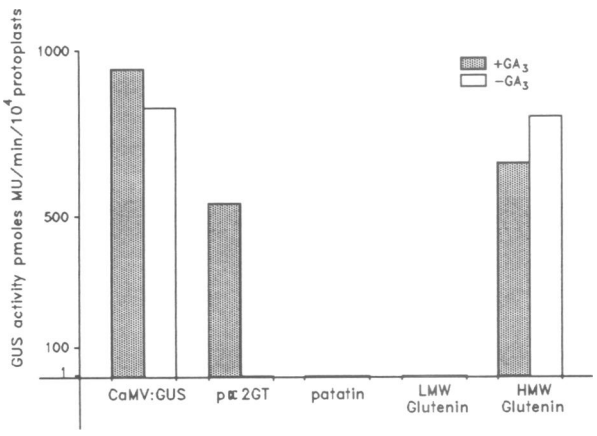

Fig. 4. Transient expression analysis of five promoter constructs in oat aleurone protoplasts. Plasmids containing transcriptional fusions of promoter fragments from a patatin gene (λpat21; Bevan *et al.*, 1986) low mol. wt glutenin (Colot *et al.*, 1987) and high mol. wt glutenin subunit 12 (Thompson *et al.*, 1985) to *GUS* were transformed into oat aleurone protoplasts and transient expression analysed after 5 days. CaMV:GUS and pα2GT were transformed as controls. Following transformation protoplasts were divided and incubated in the presence or absence of 10^{-6} M GA_3.

expression varied by as much as 10-fold. In the experiments in Figure 5 the large number of constructs made it necessary to combine the data of several experiments. This was achieved by using the GA_3-stimulated expression from pα2GT as a standard, as described in the text.

Transient expression using pα2GT

Following optimization of the transformation protocol and GUS assay with CaMV:GUS, the *α-Amy2/54* promoter in the plasmid pα2GT (Figure 1) was introduced into oat aleurone protoplasts. This promoter also directed high levels of expression, but only when the transformed protoplasts were incubated in the presence of GA_3 (Figure 2, Table I). Maximal expression of GUS was obtained when 10^{-7} M GA_3 was included in the incubation media (Figure 2) but a significant level of GUS activity could still be detected with 10^{-10} M GA_3. Protoplasts from the same transformation but incubated in the absence of GA_3 produced little or no GUS activity. Over the course of 20 independent experiments expression from GUS in those protoplasts incubated in the absence of GA_3 was between 0.5 and 3% of the level obtained from the same number of transformed protoplasts incubated in the presence of 10^{-6} M GA_3. Expression from the *α-Amy2* promoter paralleled that of the endogenous oat α-amylase genes, which was monitored by measuring the amount of α-amylase secreted from transformed protoplasts into the incubation media (Figure 2). No α-amylase activity was thus detected when transformed protoplasts were incubated in the absence of GA_3 and increasing amounts produced as the concentration of GA_3 in the media was raised until a similar maximum was reached at $\sim 10^{-7}$ M GA_3.

The antagonistic effect of ABA on α-amylase expression in aleurone cells is well documented (Nolan *et al.*, 1987). Table I shows that inclusion of 2.5×10^{-5} M ABA in the incubation media together with 10^{-6} M GA_3 directly after transformation reduced the level of endogenous oat α-amylase expression to that of GA_3-untreated controls. ABA similarly abolished GA_3-stimulated expression of GUS by those protoplasts transformed with pα2GT.

The control of expression by GA_3 and ABA as described above for the wheat α-amylase promoter was not due to a general feature of the transient assay system as expression found to be independent of the inclusion of GA_3 in the incubation media and unaffected by the addition of ABA (Table I).

In wild oat aleurone protoplasts, expression of α-amylase was characteristically not detected until after 2 days

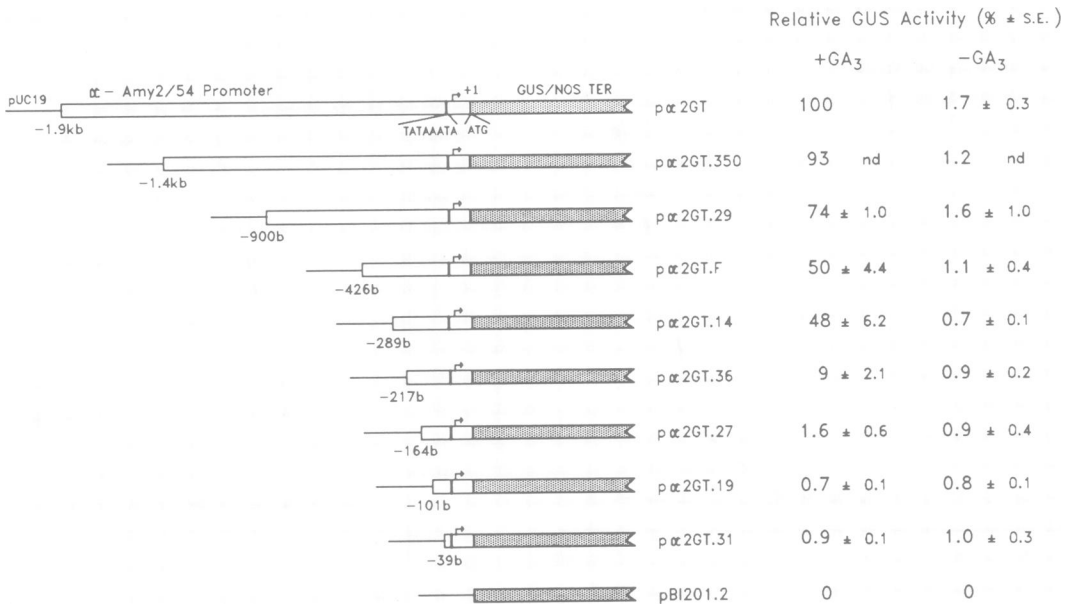

Fig. 5. Diagram showing the series of 5′ deletions constructed from pα2GT and their relative ability to direct the expression of GUS. Following transformation with equimolar quantities of plasmid DNA, protoplasts were divided into two and incubated with or without 10^{-6}M GA_3. GUS activity was then assayed after 5 days. pBI201.2 lacks any upstream promoter sequences containing only the *GUS* gene and *NOS TER*. S.E., standard error of means; nd, not determined.

incubation in the presence of GA_3 (Hooley, 1982). The same delay in expression of endogenous oat α-amylase was also observed in transformed cultivated oat protoplasts (Figure 3A). Figure 3B shows the time course of GUS expression from protoplasts transformed with pα2GT or CaMV:GUS. No GUS activity was detected from protoplasts transformed with pα2GT for 2 days following transformation and high levels only after 4−5 days of incubation in media containing GA_3. Expression from the *α-Amy2/54* promoter thus followed closely that of the endogenous α-amylase genes. A quite different pattern of GUS expression was observed from those protoplasts transformed with CaMV:GUS. In this case high levels of GUS activity were produced much earlier, <24 h after transformation. The results presented in Figure 3 show expression directed by CaMV:GUS only when the protoplasts were incubated in the presence of GA_3; the absence of GA_3, however, had no effect on the temporal expression of GUS when directed by CaMV:GUS (data not shown).

From the results presented in Table I and Figures 2 and 3 it was concluded that GA_3 was specifically controlling the transient expression from the wheat *α-Amy2* promoter and in a manner that was very similar to its control of the endogenous oat α-amylase genes.

Transient expression from additional promoters

In addition to pα2GT and CaMV:GUS, constructs containing the promoters of a patatin gene, a low mol. wt glutenin and high mol. wt glutenin subunit 12 gene as transcriptional fusions to *GUS* were also introduced into the oat aleurone protoplasts. Patatin is expressed in potato tubers (Mignery *et al.*, 1984) while both the glutenin genes are normally active in the endosperm of developing wheat grains (Bartels and Thompson, 1986). Transformation of protoplasts with chimeric constructs of patatin and low mol. wt glutenin promoters resulted in little or no detectable GUS activity being produced; the high mol. wt glutenin promoter, however, was highly active (Figure 4). Expression from the high mol. wt glutenin promoter was not significantly affected by the presence or absence of GA_3 and so was similar to that directed by the CaMV 35S promoter (Figure 4, Table I). This experiment thus reconfirmed the specificity of the GA_3 response by the wheat α-amylase promoter in this transient assay system.

Table II. Effects of GA_3 and ABA on the transient expression of *GUS* from pα2GT, pα2GT.F and pα2GT.14

Construct	β-Glucuronidase activity[a]			Oat α-amylase[b]		
	$-GA_3$	$+GA_3$	$+GA_3$ +ABA	$-GA_3$	$+GA_3$	$+GA_3$ +ABA
pα2GT	1.7	85	1.8	0.1	11.4	0.2
pα2GT.F	1.2	40	2.1	0.2	10.7	0.2
pα2GT.14	0.3	33	2.3	0.1	8.8	0.2

Protoplasts following transformation were divided into three and incubated with no GA_3, 10^{-6} M GA_3 or 10^{-6} M GA_3 + 2.5×10^{-5} M ABA for 5 days before analysis. Production of endogenous oat α-amylase by the transformed protoplasts is also indicated.
[a]pmol MU/min/10^4 protoplasts.
[b]Units relative to barley malt α-amylase.

Analysis of 5′ deletions in the α-Amy2/54 promoter

In order to identify regions of the *α-Amy2* promoter involved in the regulation of expression by GA_3 a series of promoter deletions was generated. The resultant constructs were then analysed by transient expression in aleurone protoplasts. The results in Figure 5 show expression of each construct relative to pα2GT, which was transformed as a control on each occasion together with pBI201.2, which lacks any promoter sequences. Each construct was analysed a minimum of three times with the exception of pα2GT.350. Expression from the deleted promoters was progressively lower as more of

the upstream sequence was removed until position −289 (pα2GT.14) upstream of the start of transcription was reached. This construct still retained in the presence of GA_3 48% of the expression from the full-length construct. Deletion of the next 72 bp (to position −217, pα2GT.36), produced a more dramatic drop in the level of GA_3-induced expression to only 9% of that obtained from pα2GT while removal of a further 125 bp to −164 (pα2GT.27) reduced expression to those levels obtained from transformed protoplasts when incubated in the absence of GA_3. All subsequent deletions gave only this basal level of expression with the exception of the construct containing no promoter sequences (pBI201.2) from which no detectable GUS activity was obtained.

Expression from the promoter deletions in pα2GT.F and pα2GT.14 was still subject to control by both GA_3 and ABA (Table II). The deletion analysis thus suggested that the 5′ border of a region or regions important for high-level, hormonally regulated expression lies between 300 and 200 bp upstream of the start of transcription of the α-*Amy2/54* gene.

Discussion

Transient expression analysis in cultured mammalian cells has proved a valuable tool for the analysis of promoters and the DNA binding proteins associated with them (for examples see Heard *et al.*, 1987; Evans, 1988; Ptashne, 1988). Plant protoplasts can rapidly start to produce cell walls and divide in culture, thereby indicating a change in the specific cellular control processes present. For this reason, analysis of tissue or developmentally regulated promoters in plant protoplasts has been questioned. However, regulated expression from a maize alcohol dehydrogenase promoter (AdH1) in response to anaerobism in maize protoplasts has been described (Howard *et al.*, 1987) and more recently hormonally controlled transient expression of a wheat embryo gene (Em) transformed into protoplasts derived from rice suspension cultures has been shown (Marcotte *et al.*, 1988). In this paper we describe a transient assay system that will allow a detailed dissection of *cis*-acting regulatory elements in the promoters of α-amylase and other GA_3-responsive genes. Following PEG-mediated uptake of DNA, cultivated oat aleurone protoplasts retained the ability to respond to GA_3 in a manner that was similar to wild oat protoplasts and intact aleurone cells (Hooley, 1982; Figures 2 and 3, Table I). Transient expression from the wheat α-*Amy2/54* promoter in these protoplasts was shown to be specifically controlled by GA_3 and in parallel with expression of the endogenous oat α-amylase. As the transformed DNA is not likely to be chromosomal it suggests therefore that a major part of the GA_3-responsive regulation of α-amylase gene expression is not affected by chromosomal structure or DNA methylation.

A total of four other promoters were introduced into the aleurone protoplasts, two of which were also highly active. The CaMV 35S promoter directed high levels of GA_3-independent GUS activity and was used to optimize the transformation procedure. This promoter has been shown to be expressed in a wide range of plants and tissues (Jefferson *et al.*, 1987; Odell *et al.*, 1988), and it thus appears to function without a requirement for tissue-specific transcription factors. The promoter from a patatin gene was inactive in the transient assay, but as it is normally only expressed in potato tubers it is not very surprising to find that aleurone protoplasts lack the factors required to express such a promoter. The low mol. wt glutenin promoter used was also inactive, while the high mol. wt glutenin subunit 12 promoter was able to direct high-level GA_3-independent expression similar to that from the CaMV 35S promoter. The products of the high mol. wt and low mol. wt glutenin genes are wheat storage proteins that are deposited in protein bodies in the starchy endosperm cells of developing cereal grains (Miflin *et al.*, 1981); consistent with this, expression of high and low mol. wt glutenin genes has only been found in the developing endosperm (Bartels and Thompson, 1986). The aleurone layer is a part of the endosperm of cereals (Evers, 1970), but while some storage proteins are expressed in developing aleurone cells (Mundy and Fincher, 1986), high and low mol. wt glutenins are not thought to be among them, although for high mol. wt glutenins this has not been shown. It is also generally believed that expression of these proteins does not occur in mature aleurone cells in germinating grain. The two storage protein promoter constructs have been transferred to tobacco via *Agrobacterium* transformation (Colot *et al.*, 1987) and were shown to contain sufficient DNA to exhibit expression that was confined to developing tobacco endosperm; tobacco does not, however, have a tissue analogous to the aleurone layer. In conclusion, expression from a high mol. wt glutenin promoter could represent a lack of correct transcriptional control by the aleurone protoplasts. If so, further work would be necessary to determine whether this was due to a change in the state of differentiation of the protoplasts during isolation or culture resulting in the presence of abnormal positive factors or absence of normal negative ones, or whether it could be that expression from the high mol. wt glutenin promoter was due to a direct consequence of presenting aleurone protoplasts with naked DNA, implying that normal control of the high mol. wt glutenin promoter requires a process in addition to *trans*-acting factors, such as DNA methylation or chromosomal condensation.

Expression from both the CaMV 35S and high mol. wt glutenin promoters was not subject to control by GA_3; hence this response was specific to the introduced wheat α-*Amy2* promoter. Also specific to the pα2GT promoter and endogenous α-amylase genes was the long delay of 2 days before expression could be detected. Expression of GUS directed by the CaMV:GUS construct during this lag phase indicates that the protoplasts were highly competent in gene expression between 0 and 2 days, thus the latent period cannot merely be a delay in reinstatement of the transcription apparatus as the aleurone cells recover from dehydration. It seems more likely that this is a period during which inhibitors of the GA_3 response are inactivated or mediators are activated or synthesized. It must, however, be pointed out that because the plasmid pα2GT contains a translational fusion of α-*Amy2/54* to *GUS*, the delay in expression and GA_3 control could be due in part to translational control by the α-*Amy2/54* transcribed but untranslated leader sequence. Zwar and Hooley (1986) have shown, though, that levels of oat α-amylase mRNA increase in parallel with α-amylase activity in oat aleurone protoplasts and experiments analysing nuclear run-off in wild oat and barley protoplasts suggest that GA_3 induces an increase in the rate of transcription of the α-amylase genes (Jacobsen and Beach, 1985; Zwar and

Hooley, 1986). Construction of a transcriptional fusion between *α-Amy2/54* and *GUS* could be used to elucidate this question.

The series of 5′ deletions in the *α-Amy2* promoter indicated that those regions important for high-level hormonally regulated expression lie within 300 bp upstream of the start of transcription. Reduction in the levels of expression of the constructs, containing promoter fragments of between 1400 and 300 bp, relative to pα2GT, may indicate the removal of specific enhancer sequences in these regions, although the slow, progressive nature of the reduction might be more indicative of a non-specific effect due to the replacement of *α-Amy2/54* sequence with pUC19. Sequence analysis showed that the promoter fragment in pα2GT.14, retaining 48% of the level of expression of pα2GT, just contained all of the regions of highly conserved sequence found upstream of the start of transcription of *α-Amy2* genes from wheat and barley (Huttly *et al.*, 1988). These conserved sequences have been implicated as important for expression as the only isolated gene found to lack them, the wheat gene *α-Amy2/46*, was also shown to be very poorly expressed, if at all. 5′ deletions extending into this conserved region dramatically reduced then rapidly abolished expression in transformed protoplasts to a level similar to that observed when protoplasts were incubated in the absence of GA_3. Retention of a low level (9%) of GA_3-regulated expression by pα2GT.36 could be explained in one of two ways: it might indicate the removal of a non-specific enhancer located in the region between the deletions in pα2GT.14 and pα2GT.36 (−289 and −217); or, equally, partial disruption of a sequence around −217 that is responsible for both high-level expression and/or GA_3 regulation. The GA_3-induced binding of a tissue-specific nuclear factor to the upstream regions of a rice *α-Amy2* type gene has been reported (Ou-Lee *et al.*, 1988). The 3′ end of a fragment protected by the bound factor was determined to be −143 bp from the start of transcription of that gene. Precise alignment of sequences between rice and wheat genes is not possible but is sufficient to suggest that a similar factor might bind between the deletions contained in pα2GT.36 and pα2GT.19 (−217 and −101), which is consistent with the data described above.

The mechanism of action of ABA on α-amylase expression is thought to be at the level of transcription, similar to that of GA_3 (Zwar and Hooley, 1986). The deletion analysis showed that similar regions of the promoter were involved in ABA control, since GA_3-induced expression from pα2GT.14 was still abolished by ABA, but the analysis was not sufficiently detailed to show whether ABA and GA_3 exert this control through the same regulatory element.

Materials and methods

Isolation of aleurone protoplasts

Aleurone protoplasts were isolated from mature grain of the cultivated spring oat *A.sativa* var. Rhiannon. The preparation of aleurone layers and isolation of protoplasts was carried out according to the procedure of Hooley (1982) for wild oat grain.

DNA transformation of protoplasts

Washed protoplasts were resuspended in high salt buffer (125 mM NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 5 mM glucose, 125 mM $CaCl_2$, 25 mM $MgCl_2$, pH 5.3) and mixed for 10 min. They were then collected by centrifugation for 1 min at 50 *g*, washed twice with M9 incubation media [Gamborg's B-5 medium containing 0.4 M mannitol, 2% (w/v) glucose, 10 mM L-arginine, 20 mM $CaCl_2$, pH 5.4] and finally resuspended in M9 at $5-10 \times 10^5$ protoplasts per ml and divided into 200 μl aliquots. Plasmid DNA (10−20 μg) was added to the protoplasts together with 30−40 μg of carrier DNA (sheared calf thymus DNA) in a total volume of 50 μl. This was mixed gently and 350 μl of 25% (w/v) PEG 1500, 0.1 M $Ca(NO_3)_2$, 0.45 M mannitol, pH 9, added and mixed. The protoplasts were left at room temperature for 20 min with occasional mixing. After this time 5 ml of 0.2 M $CaCl_2$, pH 6, was added in steps of 1 ml with 5 min between additions. The protoplasts were collected by centrifugation for 1 min at 50 *g* and washed twice in 2 ml of M9. They were then divided equally into two, and each aliquot was resuspended in 2 ml of M9 containing 10 μg/ml chloramphenicol, 25 U/ml nystatin and 20 μg/ml ampicillin. To one aliquot GA_3 was added to 10^{-6}M unless stated and both samples were incubated in the dark at 25°C in 3 cm Petri dishes.

β-Glucuronidase assay

Transient expression was assayed 1−5 days after transformation. Protoplasts were collected by centrifugation for 1 min at 50 *g* then resuspended in 2 ml of 0.4 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.4. A 100−200 μl aliquot of protoplasts was taken and live protoplasts counted using a haemocytometer and the vital stain methylene blue (0.01%). The remainder were collected by centrifugation (1 min, 50 *g*) then resuspended in 150 μl of GUS buffer (50 mM Na_3PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM DTT) containing 5−10 mg of acid-washed Polyclar AT (BDH). The protoplasts were lysed by vortexing briefly then left to stand for 10 min, before the suspension was placed on a 1 ml column of Sephadex G25 equilibrated in GUS buffer and packed in a 1.5 ml Eppendorf tube by centrifugation for 4 min at 450 *g*. The extract was then centrifuged through the packed column for 4 min at 450 *g* and a further 50 μl of GUS buffer centrifuged through the column for 4 min. The eluted fractions were pooled and made up to 250 μl with GUS buffer and aliquots of 50 μl were taken for analysis. β-Glucuronidase (GUS) activity was assayed by addition of 250 μl of GUS buffer containing 4-methylumbelliferyl β-D-glucuronide (MUG; final concentration 2 mM) to the aliquots. Assays were placed at 37°C and allowed to equilibrate for 10 min. A 50 μl aliquot was then removed, and at hourly intervals thereafter, into 200 μl of 1 M Na_2CO_3 in order to terminate the reaction and increase the fluorescence from 4-methylumbelliferone (MU). Fluorescence was measured in the stopped reactions using a microtitre plate reader (Fluroskan) as described by Jefferson (1987) and activity expressed as pmol MU/min/10^4 live protoplasts.

α-Amylase assay

α-Amylase activity was assayed from the supernatant medium of transformed protoplasts by the method of McCleary (1980) using alphachrome (Koch-Light). Incubations were carried out for 1 h at 37°C or 15 h at 30°C and quantified relative to barley malt α-amylase (Sigma).

DNA manipulations

All DNA constructs were cloned in the vector pUC19 using general molecular biological methods (Maniatis *et al.*, 1982). DNA was prepared from 400 ml cultures of *Escherichia coli* TG2 F′(*tra*D36, *pro*AB, *lacI*^{Q}, *lacZ*ΔM15), Δ(*lac,pro*), *Sup*E, *thi*, *recA*, *SrI::Tn10*^{TcR}, *hsd*Δ5 (r^-,m^-) and banded twice in CsCl gradients. Plasmid DNA was finally resuspended at 2 mg/ml in TE buffer and stored in aliquots at −20°C.

Construction of plasmid pα2GT

A translational fusion between the α-amylase gene *α-Amy2/54* and the reporter gene *GUS* present in the expression cassette pBI201.2 was constructed as outlined in Figure 1. pBI201 contains a promoter-less *GUS* gene fragment cloned into the *Kpn*I site in the polylinker of pUC19 such that it is possible to construct translational fusions by insertion into the remaining restriction sites in the polylinker (*Sma*I to *Hin*dIII). The cassette also contains the polyadenylation signal from the nopaline synthase gene (*NOS TER*) located 3′ to the *GUS* fragment. The first exon of α-amylase genes encodes a signal sequence capable of directing the secretion of α-amylase from aleurone cells. It was therefore necessary to construct a translational fusion containing only a small portion of the *α-Amy2/54* first exon so as to prevent secretion of the fusion protein out of the oat aleurone protoplasts during transient expression. To do this an *Sph*I site was introduced into the *α-Amy2/54* sequence at the initiating methionine codon of the gene. This was achieved by site-directed mutagenesis (Carter *et al.*, 1985) on a 400 bp fragment of *α-Amy2/54* which spanned the start of translation cloned in M13mp8, with the oligonucleotide 5′-CGTTCTTCCGCATGCCGT-ACG-3′ synthesized by A.Northrop (IAP, Babraham). Using the introduced site, a 100 bp *Bal*I−*Sph*I fragment was inserted into pUC19; the remainder of the *α-Amy2/54* sequence on a 1.9 kbp *Hin*dIII−*Bal*I fragment was then

placed upstream. The *GUS* gene and *NOS TER* from pBI201.2 were inserted into the same construct using *Sph*I and *Eco*RI sites. Within the polylinker of pUC19 there is a single termination codon (TAG) contained within the *Xba*I site. Since this termination codon was in-frame with the *α-Amy2/54: GUS* fusion formed above, it was necessary to remove this part of the polylinker. This was achieved by digesting the fusion with *Hinc*II and *Sma*I and re-ligating. This created the plasmid pα2GT, which encodes a β-glucuronidase fusion protein containing 10 extra amino acids at the N terminus, including the initiating methionine codon of *α-Amy2/54*. Additional N-terminal sequences have been shown to have little or no effect on GUS activity (Jefferson, 1987). Before use, pα2GT was checked by sequencing around the junctions and site of mutation.

Additional constructs

The transcriptional fusion of a 800 bp fragment from the CaMV 35S RNA promoter to *GUS* with the *NOS* polyadenylation signal in pUC19 (pBI221.1) was a gift of R.Jefferson (Jefferson, 1987). Transcriptional fusions of a 1 kbp promoter fragment from a patatin gene (λpat21), a 1.4 kbp fragment from high mol. wt glutenin subunit 12 gene and a 1 kbp low mol. wt glutenin promoter fragment to *GUS:NOS TER* were gifts from M.Bevan, L.Roberts and V.Colot respectively. These promoters were all tested previously for expression in transgenic plants and found to contain sufficient information to direct high-level, tissue-specific expression (Colot *et al.*, 1987; Jefferson, 1987; Jefferson *et al.*, 1987).

Promoter deletions of pα2GT

The plasmid pα2GT was cut with *Hin*dIII and digested with exonuclease III. Aliquots were taken from the reaction at regular intervals and the single-stranded region removed with Mung bean nuclease. To liberate fragments containing a deleted *α-Amy2/54* promoter fused to *GUS:NOS TER* from the vector pUC19, the DNA was restricted by *Eco*RI and *Dra*I. The fragments generated were isolated from agarose gels and recloned in pUC19 cut with *Eco*RI and *Sma*I. Clones from each time point of the exonuclease III digestion were isolated and sized on polyacrylamide gels, then sequenced using a double-stranded sequencing protocol (Murphy and Kavanagh, 1988) to determine their end points accurately.

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