

Heterotrimeric G Proteins Are Implicated in Gibberellin Induction of α -Amylase Gene Expression in Wild Oat Aleurone

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The role of heterotrimeric G proteins in gibberellin (GA) induction of α -amylase gene expression was examined in wild oat aleurone protoplasts. Mas7, a cationic amphiphilic tetradecapeptide that stimulates GDP/GTP exchange by heterotrimeric G proteins, specifically induced α -amylase gene expression and enzyme secretion in a very similar manner to GA₁. In addition, Mas7 stimulated expression of an α -Amy2/54:GUS promoter and reporter construct in transformed protoplasts. Both Mas7 and GA₁ induction of α -amylase mRNA were insensitive to pertussis toxin. Hydrolysis-resistant nucleotides were introduced into aleurone protoplasts during transfection with reporter gene constructs. GDP- β -S, which inhibits GDP/GTP exchange by heterotrimeric G proteins, completely prevented GA₁ induction of α -Amy2/54:GUS expression, whereas GTP- γ -S, which activates heterotrimeric G proteins, stimulated expression very slightly. Novel cDNA sequences from G $_{\alpha}$ and G $_{\beta}$ subunits were cloned from wild oat aleurone cells. By using RNA gel blot analysis, we found that the transcripts were expressed at a low level. Heterotrimeric G proteins have been implicated in several events during plant growth and development, and these data suggest that they may be involved in GA regulation of α -amylase gene expression in aleurone.

INTRODUCTION

Seed reserve mobilization in the Gramineae is coordinated to a large extent by aleurone cells, which are regulated by the opposing actions of the plant hormones gibberellin (GA) and abscisic acid (ABA) (Hooley, 1994). GA is perceived at the aleurone plasma membrane (Hooley et al., 1991; Gilroy and Jones, 1994) and induces transcription of α -amylase genes. This effect is overcome by ABA (Jacobsen and Beach, 1985; Zwar and Hooley, 1986). Functionally important DNA sequence elements in the promoters of GA- and ABA-regulated α -amylase genes have been identified, and genes encoding proteins that bind to some of these have been cloned (Gubler et al., 1995; Rushton et al., 1995). A GA-regulated *myb* gene, *GAmyb*, encodes a protein that binds to the GA response element of high-pl α -amylase promoters. *GAmyb* is thought to be a component of the GA response pathway and has been shown to transactivate a high-pl α -amylase promoter (Gubler et al., 1995). It is not known how GA regulation of *GAmyb* and α -amylase gene transcription is linked to GA perception at the plasma membrane. However, recent evidence has suggested the possible involvement of protein phosphatases (Kuo et al., 1996),

cGMP (Penson et al., 1996), and inositol 1,4,5-trisphosphate (Xiong et al., 1997) at early stages in GA signal transduction.

Heterotrimeric G proteins are associated with the cytoplasmic face of the plasma membrane of a variety of eukaryotic cells and transduce information from cell surface G protein-coupled receptors to effector proteins of signal transduction pathways (Neer, 1995). Microsomal and plasma membranes from a number of plant species have been shown to contain high affinity α -³²P-GTP and ³⁵S-GTP- γ -S binding activity along with polypeptides that can be ADP-ribosylated or that cross-react with antisera raised against G $_{\alpha}$ subunits or G $_{\alpha}$ subunit peptides (reviewed in Ma, 1994). Genomic and cDNA clones encoding a single class of G $_{\alpha}$ subunits and a G $_{\beta}$ subunit have been isolated from several plant species (Ma et al., 1990, 1991; Poulsen et al., 1994; Weiss et al., 1994; Ishikawa et al., 1995; Kim et al., 1995; Seo et al., 1995). Currently, it is not known whether the cloned G $_{\alpha}$ and G $_{\beta}$ subunit cDNAs encode proteins that are functional homologs of their mammalian and non-plant counterparts. Nevertheless, evidence for the involvement of heterotrimeric G proteins in signal transduction pathways in higher plants is accumulating from several studies.

A wealth of evidence indicates a role for G proteins in the regulation of K⁺ influx channels of stomatal guard cells (Assmann, 1996). The guanine nucleotide analogs, GTP- γ -S and GDP- β -S, cholera toxin and pertussis toxin (PTX), and

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the G protein agonist Mas7 have been shown to modulate inward K^+ channel activity of guard cells in a complex manner that may indicate multiple G protein signaling pathways in guard cells (Fairley-Grenot and Assmann, 1991; Lee et al., 1993; Li and Assmann, 1993; Wu and Assmann, 1994; Armstrong and Blatt, 1995; Kelly et al., 1995). Similar studies have implicated G proteins in responses to plant hormones (Zaina et al., 1990; Bossen et al., 1991), blue and red light (Warpeha et al., 1991; Neuhaus et al., 1993; Romero and Lam, 1993; Bowler et al., 1994), pathogen resistance and pathogen-related gene expression (Beffa et al., 1995), and fungal elicitors (Legendre et al., 1992).

In this investigation, we assessed the role of heterotrimeric G proteins in signal transduction in GA-responsive aleurone protoplasts of wild oat. Using synthetic peptides, PTX, and nucleotide analogs, we obtained evidence that suggests a role for heterotrimeric G proteins in signal transduction leading to α -amylase gene expression in wild oat aleurone. In addition, we describe the cloning, sequencing, and expression of G_α and G_β subunit sequences from wild oat aleurone.

RESULTS

Mas7 Induces α -Amylase Production and Secretion by Aleurone Protoplasts

To examine the possible involvement of heterotrimeric G proteins in signal transduction in wild oat aleurone, we incubated aleurone protoplasts with the mastoparan analog Mas7. Mas7 is a cationic amphiphilic tetradecapeptide that stimulates GDP/GTP exchange by heterotrimeric G proteins (Higashijima et al., 1988, 1990). Mas7 has been used as a probe of heterotrimeric G protein action in plant cells (White et al., 1993; Armstrong and Blatt, 1995; Cho et al., 1995; Scherer, 1995; Crespi et al., 1996; Tucker and Boss, 1996; Gelli et al., 1997).

Aleurone protoplasts produced and secreted α -amylase in response to Mas7. Figure 1A shows that the response was dose dependent, with concentrations as low as 0.1 μ M producing a significant response compared with untreated controls. The optimum response occurred at between 3 and 4 μ M, with a sharp decline at higher concentrations of Mas7. Vital staining with methylene blue at the end of the incubation period revealed that viability of protoplasts was not affected by up to 3 to 4 μ M Mas7 but that higher concentrations were toxic to protoplasts, as shown in Figure 1B. Non-specific detrimental effects on membrane function are known to be caused by high concentrations of mastoparan (Weidman and Winter, 1994), and we assume that this is the reason for the reduction in protoplast survival at high concentrations of Mas7. An inactive mastoparan analog, MasCP (control peptide) differing from Mas7 by a single amino acid substitution, did not induce α -amylase and was not toxic to protoplasts. Mas7-COOH was also inactive. It is a peptide with the same amino acid sequence as Mas7, but a free acid

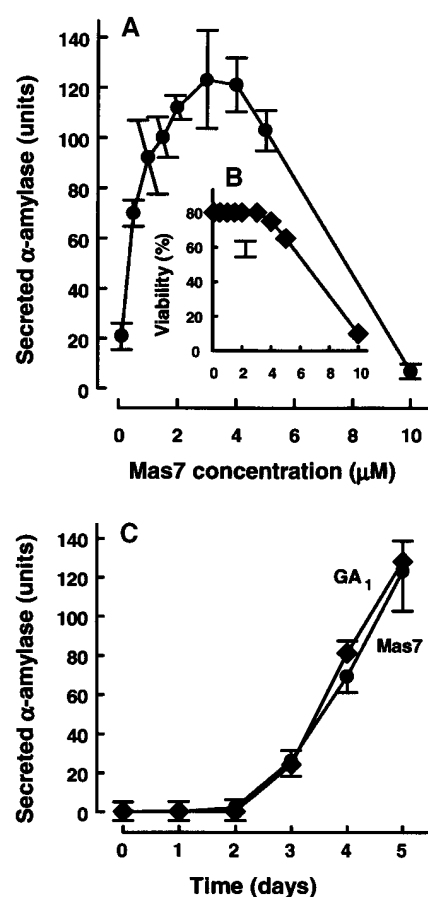


Figure 1. Mas7 Induction of α -Amylase in Wild Oat Aleurone Protoplasts.

(A) Aleurone protoplasts were incubated for 5 days with a range of concentrations of Mas7, and the amount of secreted α -amylase was determined (closed circles). Bars indicate standard error of three replicate experiments. Control protoplasts incubated without Mas7 secreted 2 ± 1 units of α -amylase. Protoplasts incubated with a range of concentrations of either MasCP or Mas7-COOH secreted 2 ± 1 units of α -amylase.

(B) Protoplast viability was measured at the end of the incubation period (closed boxes). Bar indicates the largest standard error of the points determined in three replicate experiments. Protoplast viability was unaffected by any concentration of MasCP.

(C) Aleurone protoplasts were incubated for various times with either 0.1 μ M GA₁ (closed boxes) or 3 μ M Mas7 (closed circles), and the amount of secreted α -amylase was determined. Bars indicate standard error of three replicate experiments. Control protoplasts incubated without GA₁ or Mas7 had secreted 2 ± 1 units of α -amylase at day 5. Protoplasts were incubated for 5 days with 1.0 μ M ABA and either 0.1 μ M GA₁ or 3 μ M Mas7, and they secreted 10.2 ± 0.8 and 8.9 ± 1.4 units of α -amylase, respectively.

replaces the amine group at the C terminus. These data are consistent with the activity of Mas7 and its derivatives in mammalian cells.

We compared the time course and magnitude of α -amylase induction by optimal concentrations of Mas7 (3 μ M) and GA₁ (0.1 μ M). Figure 1C shows that Mas7 and GA₁ stimulated α -amylase production and secretion with a virtually identical time course and that by 4 and 5 days, very similar amounts of α -amylase had been secreted in response to each of the treatments. ABA (1.0 μ M) largely overcame both Mas7- and GA₁-induced secretion of α -amylase. The same pattern of six α -amylase isoenzymes between pI 4.4 and 5.1 was induced by Mas7 and GA₁ treatments (data not shown). It was also possible to induce α -amylase with Mas7 in isolated aleurone layers. For example, Mas7 (3.5 μ M)-treated wild oat aleurone layers secreted 136 ± 25 units of α -amylase after 5 days. Aleurone layers treated with mastoparan (6.4 μ M) secreted 77 ± 11 units of α -amylase after 5 days.

Mas7 Stimulates α -Amylase Gene Transcription

To examine whether Mas7 induces α -amylase gene transcription, we conducted RNA gel blot analyses and transient expression assays. Gel blot analysis of total RNA from aleurone protoplasts revealed that Mas7 stimulated α -amylase gene expression but was less effective than GA₁. PTX had little or no effect on the amount of α -amylase transcript in the untreated controls or in the Mas7- or GA₁-treated protoplasts, as shown in Figures 2A and 2B.

Aleurone protoplasts were transformed with the α -amylase- β -glucuronidase α -Amy2/54:*GUS* construct p α 2GT (Huttly and Baulcombe, 1989) and treated with GA₁, Mas7, or MasCP. Figure 2C shows that Mas7 (3 μ M) and GA₁ (50 nM) induced *GUS* expression to levels significantly higher than the MasCP (3 μ M) or minus GA controls. Mas7 was not as effective at inducing *GUS* expression as GA₁. Neither Mas7 nor GA₁ had any effect on *GUS* expression in protoplasts transformed with the cauliflower mosaic virus (CaMV):*GUS* plasmid (Huttly and Baulcombe, 1989), as shown in Figure 2D. Taken together, these data demonstrate that Mas7 induces α -amylase gene expression and α -amylase secretion by aleurone protoplasts and suggest that if Mas7 is acting through a heterotrimeric G protein, the G α subunit of this G protein is probably insensitive to PTX.

Effect of Nucleotide Analogs on GA Regulation of the α -Amy2/54 Promoter

To investigate further the possible role of heterotrimeric G proteins in GA regulation of α -amylase gene transcription, we examined the effects of the hydrolysis-resistant guanine nucleotide analogs GTP- γ -S and GDP- β -S on α -Amy2/54:*GUS* expression. These nucleotides have been used to study G protein signaling in plant cells (Ma, 1994; Assmann,

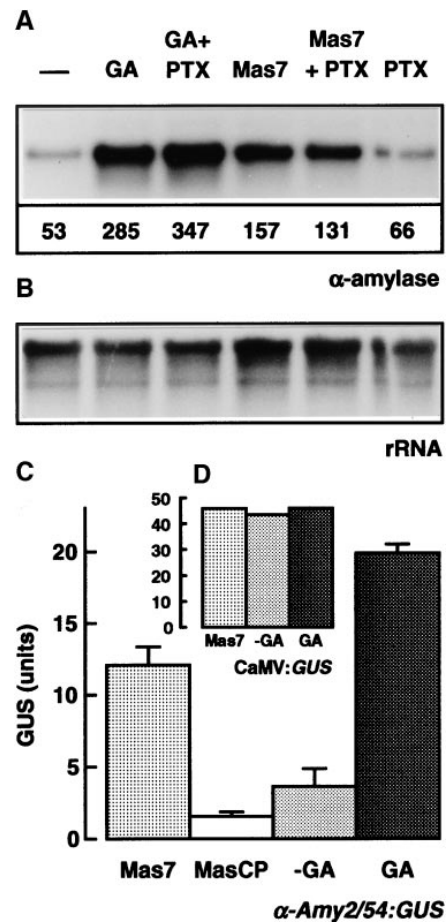


Figure 2. Mas7 Induction of α -Amylase Gene Expression in Wild Oat Aleurone Protoplasts.

(A) and (B) Gel blot analysis of total RNA (10 μ g per track) from aleurone protoplasts. In (A), the filter was probed with a 1555-bp cDNA encoding a low-pI α -amylase gene, *Afa2* (Hooley et al., 1991). In (B), the same filter was reprobed with a 1200-bp cDNA, *Afr4*, specific to wild oat 25S rRNA (Hooley et al., 1991). Freshly prepared aleurone protoplasts were treated overnight with or without PTX (1 μ g mL⁻¹) and then incubated with GA₁ (10 nM) (GA) or Mas7 (3.5 μ M) or given no further treatment (minus sign) for 5 days. Further additions of PTX (1 μ g mL⁻¹) were made at 48-hr intervals throughout the 5-day incubation. Autoradiographs were digitized using a Bio-Rad Gel Doc 1000 system, and the volume integration value for each band was determined after subtraction of local background by using Molecular Analyst Software (Bio-Rad) version 1.5. The volume integration value for each band in (A) was corrected for equal loading of rRNA and is displayed numerically in the lower part of (A).

(C) and (D) Transient expression analysis of α -Amy2/54:*GUS* and CaMV:*GUS* in aleurone protoplasts. Freshly prepared aleurone protoplasts were transformed with (C) α -Amy2/54:*GUS* or (D) CaMV:*GUS* and incubated for 5 days with Mas7 (3 μ M), MasCP (3 μ M), or GA₁ (50 nM) (GA) or without GA (-GA). *GUS* activity is presented as micromoles of 4-methylumbelliferone per minute per 10⁵ protoplasts. Bars indicate standard error of three replicate experiments. Data in (D) are means of duplicate experiments.

1996). They bind to G_α subunits and inhibit the cycle of GTP/GDP exchange and thus tend to hold the α subunit in the activated (GTP- γ -S-bound) or inactivated (GDP- β -S-bound) form. Because these nucleotides are considered to be membrane impermeant, we needed a method of introducing them into aleurone protoplasts. In addition, because GTP- γ -S and GDP- β -S can be expected to hydrolyze in vivo, albeit more slowly than GTP and GDP, it was necessary to devise an assay in which GA induction of α -*Amy2/54*:*GUS* expression could be measured more rapidly than the usual 5 days (Huttly and Baulcombe, 1989).

We overcame the latter problem by aging protoplasts for 4 days in M9 medium (Hooley, 1982) before transformation and treatment with GA. Four-day-old wild oat aleurone protoplasts respond to GA substantially quicker than do freshly isolated protoplasts (S.J. Smith, A. Lovegrove, and R. Hooley, unpublished data), and this allowed us to measure GA-induced α -*Amy2/54*:*GUS* expression after 48 hr. Under these conditions, GA-stimulated α -*Amy2/54*:*GUS* expression did not reach the levels attained in freshly isolated protoplasts treated with GA for 5 days (cf. Figures 2C and 3C). To address the former problem, we examined whether GTP- γ -S and GDP- β -S could be introduced into protoplasts during calcium-polyethylene glycol-mediated transformation with the reporter constructs. Figure 3A shows that when aleurone protoplasts were transformed in the presence of 500 μ M GTP- γ -S and 3 μ Ci of 35 S-GTP- γ -S or with 500 μ M GDP- β -S and 3 μ Ci of 35 S-GDP- β -S and then washed until no significant radioactivity above background could be detected in the supernatants, there was significant and comparable uptake of both radiolabeled nucleotides. Non-transformed control protoplasts incubated with the above nucleotides in M9 showed only very low uptake, possibly nonspecific binding, of the radiolabeled nucleotides. If we assume that the nucleotides become uniformly distributed throughout the transformed protoplasts, this amount of uptake approximates an intracellular concentration of 1 μ M.

Figure 3B shows that there was no significant effect of GA_1 , or any of the nucleotides, on *CaMV*:*GUS* expression, confirming that none of the treatments had a nonspecific effect on expression from the constitutive promoter or on *GUS* protein synthesis. Figure 3C shows that GDP- β -S reduced GA_1 -induced α -*Amy2/54*:*GUS* expression to the level of the GA -minus control. ADP- β -S had no appreciable effect on α -*Amy2/54*:*GUS* expression, indicating that the effect was specific to the guanine nucleotide (Figure 3C). GTP- γ -S very slightly stimulated α -*Amy2/54*:*GUS* expression in protoplasts incubated with and without GA_1 , compared with those that had been transformed in the absence of added nucleotide or had received ATP- γ -S (Figure 3C). These effects of both GTP- γ -S and GDP- β -S were consistently observed in replicate experiments. The experiments were also repeated using 1 mM nucleotides, and the same effects were observed (data not shown). Using 100 μ M nucleotides, however, we could detect no effects of GDP- β -S and GTP- γ -S (data not shown).

Wild Oat Aleurone Cells Express G_α and G_β Subunit Sequences

In making deductions about the possible involvement of heterotrimeric G proteins in signal transduction in aleurone, it is important to demonstrate that these proteins are likely to be present in aleurone cells. Wang et al. (1993) have reported that barley aleurone cells may contain monomeric G proteins, possibly related to Ras. We have used a molecular cloning approach to identify heterotrimeric G protein subunits in wild oat aleurone.

We designed polymerase chain reaction (PCR) primers specific to conserved regions in G_α and G_β subunits and used these to amplify products from aleurone cDNA. PCR products of the expected sizes were subcloned, sequenced, and compared with sequences in the DNA and protein sequence databases. This showed the G_α and G_β subunit sequences to be novel. The cDNA *AfG α 1* (GenBank accession number AF010476) is a partial clone of 657 nucleotides that aligned to the region between GTP binding regions 1 and 4 (Conklin and Bourne, 1993) of published G_α sequences. The deduced amino acid sequence of *AfG α 1* is 40% identical and 51% similar to Arabidopsis *GPA1* (Ma et al., 1990). Two closely related G_β sequences were obtained, and clones *AfG β 1* and *AfG β 2* were isolated by 3' and 5' rapid amplification of cDNA ends. One of these, *AfG β 1*, was sequenced completely on both strands (GenBank accession number AF033357). *AfG β 1* is 1470 nucleotides long, and the deduced amino acid sequence contains the seven repeats of the WD-40 motif characteristic of G_β subunits. It is 92% identical to the rice G_β subunit (GenBank accession number X89737) and 91% identical to the maize G_β subunit *ZG β 1* (Weiss et al., 1994). Based on comparison with these sequences, *AfG β 1* is likely to be full length. *AfG β 2* has been partially sequenced and appears to be related to but distinct from the G_β subunit, with 51% identity to *AfG β 1* over a 250-amino acid region.

RNA gel blot analysis was used to investigate expression of *AfG α 1* and *AfG β 1*. Poly(A)⁺ RNA was isolated from wild oat seeds that had been washed, surface sterilized, and imbibed in sterile water for 24 hr. The loadings were probed with 32 P-labeled *AfG α 1*, which revealed low-level expression of a 1.7-kb mRNA, as shown in Figure 4A. The levels of *AfG α 1* transcript were not affected by GA treatment (data not shown). The filter was reprobed with *AfG β 1*, revealing significant expression of a 1.7-kb mRNA, as shown in Figure 4B.

DISCUSSION

The mastoparan analog Mas7 stimulates GDP/GTP exchange by heterotrimeric G proteins and is thought to mimic ligand-activated G protein-coupled receptors (Higashijima et al., 1988, 1990). The fact that Mas7 and mastoparan stimulate GTP- γ -S binding in plasma membrane of pea, maize,

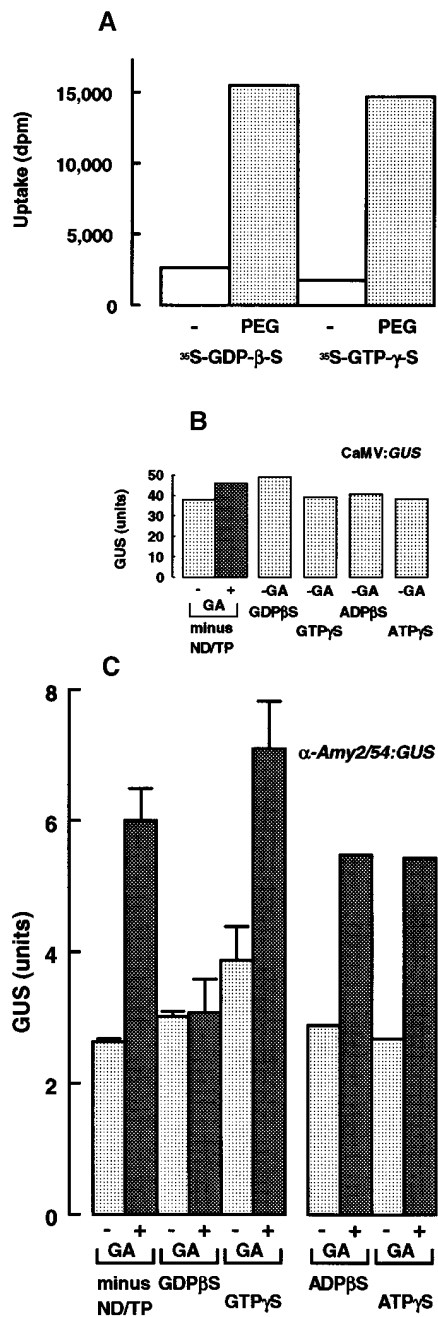


Figure 3. Effect of Hydrolysis-Resistant Nucleotide Analogs on Reporter Gene Expression.

(A) Uptake of radiolabeled nucleotides by transformed (polyethylene glycol [PEG]) and control nontransformed (-) aleurone protoplasts. Four-day-old aleurone protoplasts were transformed in the presence of 3 μCi (11.1 kBq) of $^{35}\text{S-GDP-}\beta\text{-S}$ or $^{35}\text{S-GTP-}\gamma\text{-S}$ (46.3 TBq/mmol) (Du Pont-New England Nuclear) and 500 μM GDP- β -S or GTP- γ -S. Nontransformed controls were incubated in M9 with the above-mentioned additions. Protoplasts were then washed by sedimentation through M9 medium until no significant radioactivity above background could be detected in the supernatant, counted in

and spinach suggests that they have a similar activity in plants (White et al., 1993; Crespi et al., 1996). Plant cell responses to Mas7 have suggested that heterotrimeric G proteins may be involved in the regulation of stomatal guard cell inward K^+ currents (Armstrong and Blatt, 1995), phospholipid metabolism (Cho et al., 1995), auxin stimulation of elongation growth and phospholipase A_2 activity (Scherer, 1995), intracellular Ca^{2+} fluxes (Tucker and Boss, 1996), responses to fungal elicitors (Legendre et al., 1992; Gelli et al., 1997), and red light signaling (Crespi et al., 1996).

In this study, we present evidence that Mas7 induces α -amylase gene transcription and enzyme secretion in aleurone protoplasts of wild oat and that it does so in a manner very similar to that of GA_1 . The fact that α -amylase induction was specific for the Mas7 peptide was confirmed by the observation that two control peptides, (1) MasCP, differing from Mas7 by a single amino acid substitution, and (2) Mas7-COOH, a peptide with the same amino acid sequence as Mas7 but with a free acid replacing the amine group at the C terminus, were inactive.

In addition to activating $\text{G}_{\alpha i}$ and $\text{G}_{\alpha o}$ subunits (Higashijima et al., 1990), mastoparan and its analogs have a variety of other effects on cells. These include binding to phospholipids, affecting membrane integrity, forming cation-permeable pores in the plasma membrane, and influencing Ca^{2+} -calmodulin binding and the activity of phospholipase C (Suh et al., 1996). These effects are typically brought about by concentrations of Mas7 at 10 μM and above (Suh et al., 1996). The fact that we observed significant induction of α -amylase by 0.1 and 0.5 μM Mas7 and an optimal response at 3 to 4 μM supports the theory that Mas7 may be inducing α -amylase gene expression by acting on a heterotrimeric G protein(s) in aleurone protoplasts rather than through one or more of these other effects described above. Because concentrations of Mas7 above 3 to 4 μM are detrimental to aleurone protoplast viability, it appears that Mas7 has multiple effects on aleurone protoplasts and probably affects membrane function and integrity at these higher concentrations.

Heterotrimeric G proteins are also known to be involved in secretion (Helms, 1995). It is therefore possible that Mas7 might also act on any G proteins involved in α -amylase

a hemocytometer, solubilized in OptiPhase HiSafe 3 (Wallac UK, Milton Keynes, UK), and radioactivity determined by liquid scintillation counting.

(B) and **(C)** Transient expression analysis of CaMV:GUS and α -Amy2/54:GUS in aleurone protoplasts. Four-day-old aleurone protoplasts were transformed in the presence of hydrolysis-resistant nucleotides or without nucleotides (minus ND/TP) with **(B)** CaMV:GUS or **(C)** α -Amy2/54:GUS and incubated with (+) or without (-) GA for 48 hr. GUS activity is presented as micromoles of 4-methylumbelliferone per minute per 10^5 protoplasts. Bars indicate standard error of three replicate experiments; otherwise, data are means of duplicate experiments.

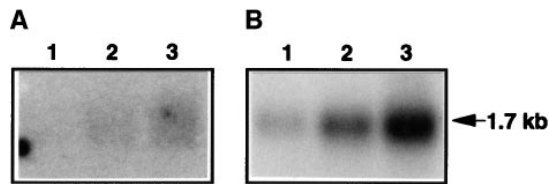


Figure 4. Gel Blots of Poly(A)⁺ RNA.

(A) Poly(A)⁺ RNA was isolated from wild oat seed that had been imbibed in water for 24 hr. Five (lane 1), 10 (lane 2), and 20 μ g (lane 3) were electrophoresed on a denaturing agarose gel, blotted onto nitrocellulose, and probed with a ³²P-random prime-labeled G _{α} cDNA. The filter was washed and exposed to Kodak Xomat-AR film for 2 weeks (Hooley et al., 1991).

(B) The filter was stored under desiccant for 3 months and reprobed using a G _{β} cDNA. Exposure time was 10 days. The transcript length is indicated at right. Similar RNA gel blots using poly(A)⁺ RNA isolated from wild oat aleurone layers detected transcripts of the same length (data not shown).

secretion and that the observed dose-response curve may be a net effect of two or more signaling mechanisms. This led us to study the effect of Mas7 and other G protein agonists/antagonists on α -amylase gene expression and on an α -amylase promoter:*GUS* reporter construct (*Amy2/54:GUS*) in transient expression experiments. These confirmed a specific effect of Mas7 on α -amylase gene transcription and suggested that if either Mas7 or GA₁ were acting through a heterotrimeric G protein, then the G _{α} subunit of this G protein was insensitive to PTX. In this context, it may be relevant that no plant G _{α} subunit cloned to date has the C-terminal cysteine residue that confers sensitivity to PTX (determined from data in GenBank).

Kuo et al. (1996) have reported that in wheat aleurone layers, 10 μ M mastoparan did not stimulate α -amylase secretion. Superficially, this observation may conflict with the data described here. However, the single concentration of mastoparan tested may have been too low or too high to elicit a response in wheat aleurone layers, or there may have been other differences in experimental conditions. Nevertheless, it is possible that in wheat aleurone, neither mastoparan nor Mas7 is capable of inducing α -amylase.

The similarity in the responses of aleurone protoplasts to Mas7 and GA₁ raises the question of whether GA₁ signals through a heterotrimeric G protein to induce α -amylase gene transcription. If this is the case, then preventing GDP/GTP exchange by G _{α} subunits should specifically block α -amylase gene transcription. By introducing hydrolysis-resistant nucleotide derivatives into aleurone protoplasts during transfection with promoter and reporter constructs, we demonstrated that GDP- β -S, which could be expected to uncouple heterotrimeric G protein signaling by inhibiting GDP/GTP exchange, completely prevented GA₁ induction of the *α -Amy2/54:GUS* reporter construct. Because ADP- β -S had no effect, inhibition was clearly specific for the guanine

nucleotide. In addition, none of the hydrolysis-resistant nucleotides tested had any appreciable effect on the expression of a constitutive promoter reporter construct (CaMV:*GUS*).

Because GTP- γ -S can be expected to constitutively activate heterotrimeric G proteins, we predict that like Mas7, it might stimulate *α -Amy2/54:GUS* expression. We observed only a slight and possibly insignificant effect of GTP- γ -S on *α -Amy2/54:GUS* expression both in the absence and presence of GA₁. This is in contrast to the strong and specific effects of Mas7 and GDP- β -S; the reasons for this are not clear. Our observations are also in contrast to the strong opposing effects of GTP- γ -S and GDP- β -S on the regulation of K⁺ channel activity in stomatal guard cells (Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993; Kelly et al., 1995). One possible explanation of our observations is that to signal effectively, heterotrimeric G proteins may need to cycle, thus providing rapidly turning over pools of GTP-G _{α} and/or G _{β} /G _{γ} complex for stimulating downstream components. Mas7 would stimulate the G protein cycle, and GDP- β -S would prevent or drastically slow it. In contrast, GTP- γ -S would significantly slow the G protein cycle and cause an accumulation of GTP- γ -S-G _{α} and G _{β} /G _{γ} complex. This may be less effective in downstream signaling for the regulation of α -amylase gene expression in the aleurone than rapidly turning over pools of these components.

Finally, we have used PCR to clone a fragment of a novel G _{α} subunit and two G _{β} subunit sequences from wild oat aleurone, and we have demonstrated that they are expressed at low levels in this tissue. This provides evidence to suggest that heterotrimeric G proteins exist in aleurone cells. These, or related sequences, may encode proteins that are the targets for the G protein agonists and antagonists used in this study.

We cannot entirely rule out the possibility that Mas7, mastoparan, and the guanine nucleotide analogs may be acting on signaling molecules other than heterotrimeric G proteins in wild oat aleurone; nevertheless, we suggest that the most plausible interpretation of our data is the one we have drawn. Heterotrimeric G proteins have been implicated in a number of events during plant growth and development (Ma, 1994; Assmann, 1996), and the results presented here suggest their involvement in GA regulation of α -amylase gene expression in the aleurone. Our observations provide additional evidence for plasma membrane-based signaling events (Hooley et al., 1991) that influence α -amylase gene expression in aleurone.

METHODS

Plant Material, Aleurone Protoplasts, and α -Amylase Assays

Seeds from a single harvest of an inbred line of wild oat (*Avena fatua*) (Hooley, 1982) were air dried and stored over anhydrous CaCl₂ for 3 years at 25 \pm 5°C. Aleurone protoplasts were prepared from them

and incubated at $1.0 \pm 0.1 \times 10^5$ per mL, as described previously (Hooley, 1982; Hooley et al., 1991), in tissue culture-quality plastic ware (Grenier Ltd., Dursley, UK). Protoplasts were aged before transformation by incubating in M9 medium (Hooley, 1982) for 4 days, as described above. They were washed twice in the incubation volume of M9 by sedimenting at 1g for 20 min, counted, and then resuspended in M9 at $\sim 1.5 \times 10^6$ mL⁻¹. Secreted α -amylase was measured as described previously (Hooley et al., 1991).

Synthetic Peptides, Pertussis Toxin, and Nucleotides

The synthetic peptides Mas7 (INLKALAALAKALL-NH₂), Mas7-free acid (INLKALAALAKALL), and MasCP (INLKAKAALAKALL-NH₂) were synthesized by the University of Bristol Molecular Recognition Centre Peptide Synthesis Service on an automatic peptide synthesizer (Milligen 9050; Millipore [UK] Ltd., Watford, UK) by using standard Fmoc chemistry with custom synthesis protocols. Peptides were purified by reverse phase HPLC on C18 Vydac columns (Vydac, Hesperia, CA) by using a gradient of water-acetonitrile containing 0.1% trifluoroacetic acid. Other samples of Mas7 and MasCP were a generous gift from P. Millner (University of Leeds, Leeds, UK). Peptides were stored in aliquots as freeze-dried powders at -20°C and prepared as aqueous solutions in silanized plastic tubes immediately before use; the peptide concentration was confirmed using the BCA assay reagent kit (Pierce and Warriner, Chester, UK). Peptides were added to aleurone protoplasts by making two times the final concentration solutions in M9 and mixing with an equal volume of protoplasts in M9 at two times the final concentration.

Pertussis toxin (PTX; Calbiochem Novabiochem Ltd., Nottingham, UK) was dissolved in water at 0.1 mg mL⁻¹. It was preactivated by adding 1 mM DTT and incubating at 30°C for 20 min. Activated PTX was added to protoplasts in incubation medium by the method described above for peptides.

Hydrolysis-resistant nucleotide analogs were obtained as lithium salts and made up at 10 mM stock solutions in water immediately before use. They were added during protoplast transformation, at the same time as DNA, to final concentrations of 100 μM , 500 μM , and 1 mM.

Transient Expression

Aleurone protoplasts were transformed with p α 2GT and cauliflower mosaic virus and β -glucuronidase (CaMV:*GUS*) plasmids (generous gifts from A. Huttly, IACR-Long Ashton), using the method of Huttly and Baulcombe (1989), with minor modifications, notably, the use of tissue culture-quality plastic ware (Grenier Ltd.) throughout and 30% rather than 25% (w/v) polyethylene glycol and for 4-day-old protoplasts, sedimentation at 1g for 20 min rather than 50g for 1 min. Promoter-driven *GUS* expression was assayed 48 hr or 5 days after transformation, as described by Huttly and Baulcombe (1989), except that after lysis, protoplasts were disrupted by sonication. *GUS* activity is expressed as micromoles of 4-methylumbelliferone per minute per 10⁵ live protoplasts.

Isolation of RNA and Gel Blot Analysis

Total RNA was isolated from aleurone protoplasts, as described previously (Hooley et al., 1991). Poly(A)⁺ RNA was isolated as follows. Total RNA was denatured at 70°C for 10 min and then quenched on

ice for 5 min. One-tenth volume of 5 M lithium chloride and 0.2 g of oligo(dT)-cellulose were added, and the mixture was swirled at 37°C for 30 min. Oligo(dT)-cellulose was pelleted at 150g for 5 min, washed twice with binding buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% [w/v] SDS, and 0.5 M LiCl), and then eluted by swirling for 5 min with hot (60°C) elution buffer (binding buffer with no LiCl). Oligo(dT)-cellulose was pelleted as given above, and the supernatant containing poly(A)⁺ RNA was removed. Oligo(dT)-cellulose was rewashed with hot elution buffer, and the RNA was pooled. The RNA was denatured a second time, and the oligo(dT)-cellulose binding/elution steps were repeated as given above. Finally, poly(A)⁺ RNA was precipitated at -20°C by the addition of a tenth volume of 3 M LiCl and 2.5 volumes ethanol, washed once with 70% ethanol, dried, and stored at -80°C .

Poly(A)⁺ RNA was isolated from 10 g dry weight of half-seeds as follows. Half-seeds were surface sterilized (Hooley, 1982) and incubated in 2 mM CaCl₂ with GA₁ (10 nM) for 4 days. Excess liquid was removed using a Buchner funnel, and the half-seeds were powdered under liquid nitrogen with a pestle and mortar. The material was warmed to -20°C and shaken vigorously for 5 min with a 1:1 mix of phenol and extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, 0.5% [w/v] SDS, pH 8.8). Phases were separated by centrifuging at 2000g for 15 min at 10°C. The aqueous phase was recovered and shaken vigorously for 5 min with an equal volume of phenol-chloroform. The phases were separated as given above, and the phenol-chloroform extraction was repeated three more times. The solvent phase was back-extracted with an equal volume of extraction buffer, which was finally pooled with the original aqueous phase before poly(A)⁺ RNA purification, as described above. The yield of poly(A)⁺ RNA was estimated spectrophotometrically. RNA gel blot analysis was performed essentially as described previously (Hooley et al., 1991). Autoradiographs were digitized using a Bio-Rad Gel Doc 1000 system and quantified using Bio-Rad molecular analyst software version 1.5.

Polymerase Chain Reaction Amplification, Cloning, and Sequencing

Double-stranded cDNA was synthesized using a cDNA synthesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Degenerate polymerase chain reaction (PCR) primers were designed and synthesized to the conserved peptide sequences AGESGK and FLNKID in G α subunits (Conklin and Bourne, 1993) and ITSSGNQTC and SGRLLFAGY of G β subunits (Weiss et al., 1994). PCRs were performed in a final volume of 50 μL on a Perkin-Elmer 480 DNA thermal cycler in thin-walled 0.5-mL Gene Amp tubes (Perkin-Elmer, Norwalk, CT). AmpliTaq DNA polymerase (0.5 units; Perkin-Elmer) was routinely used with 1 μM primers, 200 μM deoxynucleotidetriphosphates, and 1 \times manufacturer's amplification buffer supplemented with 1.5 mM MgCl₂. AmpliWax PCR Gem-100 wax (Perkin-Elmer) was used as recommended by the manufacturer to provide a hot start to the reaction. Control reactions with only one primer from each pair were performed at the same time to identify nonspecific amplification products, and reactions with no template DNA were also done to check for contamination by extraneous DNA. The thermal cycling regime used was three cycles of 94°C for 45 sec, 37°C for 45 sec, 72°C for 45 sec with a 2-min ramp, and then 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a final 72°C extension for 5 min. PCR products were ligated into pUBS3 and sequenced.

A Marathon cDNA rapid amplification of cDNA ends library (Clontech, Palo Alto, CA) was constructed using 1 μ g of poly(A)⁺ RNA, according to the manufacturer's instructions, and used to amplify the 3' and 5' ends of the G β sequences. We were not able to obtain a full-length sequence of the G α subunit cDNA.

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