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PROTEIN PHOSPHATASE INHIBITORS ENHANCE THE EXPRESSION OF AN α-AMYLASE GENE, *αAmy3*, IN CULTURED RICE CELLS

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SUMMARY: A rice (*Oryza sativa* L.) gene for α-amylase, *αAmy3*, was strongly and rapidly induced by treatment of suspension-cultured cells with okadaic acid (OA), a potent and specific inhibitor of protein serine/threonine phosphatases 1 and 2A. The massive accumulation of *αAmy3* mRNA in response to OA treatment was due to the stimulation of gene transcription and a partial stabilization of this mRNA. This induction of *αAmy3* message by OA occurred even though cellular protein synthesis was inhibited. Simultaneous treatment of cultured cells with OA and anisomycin synergistically induced *αAmy3* expression. In addition, the inhibition of protein synthesis stabilized OA-induced *αAmy3* mRNA. In the presence of protein kinase inhibitors H7, W7, and H8, *αAmy3* mRNA accumulation induced by OA was unaffected. These results indicate that OA-dependent *αAmy3* induction is regulated transcriptionally by a signal transduction pathway involving protein phosphorylation, but independent of both protein kinase C and Ca^{2+}/calmodulin- or Ca^{2+}-dependent protein kinases. Furthermore, an AMP-activated protein kinase may be required for this induction of *αAmy3* expression.

The enzyme α-amylase (EC 3.2.1.1), an endoamylase (1), catalyzes the hydrolysis of α-1,4 endoglycolytic bonds of amylose and amylopectin to convert starch into maltose and glucose. During the course of cereal seed germination, α-amylase is the most abundant hydrolase in aleurone cells, in response to gibberellin secreted from embryo, and it causes the hydrolytic breakdown of starchy endosperm to support the growth of seedlings (2, 3). Alpha-amylase gene expression is regulated both positively by gibberellic acid and negatively by abscisic acid at the transcriptional level (4, 5). In rice (*Oryza sativa* L.), α-amylase isozymes are encoded by a multigene family that contains at least ten distinct

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Abbreviations used: *αAmy* or *RAmy*, α-amylase-encoding gene; *αAmy-C*, *αAmy* cDNA clone; Bt2cAMP, dibutyryl cyclic AMP; DMSO, dimethyl sulfoxide; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide dihydrochloride; kb, kilobases or kilobase pairs; OA, okadaic acid; PCR, polymerase chain reaction; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride.

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genes classified into five hybridization groups (6, 7). Among these α-amylase genes, *RAmy3D* (8, 9) has been studied extensively.

Previous studies have shown that *RAmy3D* is expressed in all tissues except the immature seeds (9) and that the scutellar epithelium of germinated rice grains is the initial site of *RAmy3D* expression (10). Furthermore, *RAmy3D* is expressed only briefly at a low level during the early phases of germination and this mRNA reaches a peak of accumulation before 1 day's germination (10-12). However, several recent reports describe that *RAmy3D* expression is controlled by metabolic regulation. In the scutella of isolated rice embryos, the high-level accumulation of *RAmy3D* mRNA is repressed by a variety of sugars and endosperm extracts from germinated rice grains (12). In suspension-cultured rice cells derived from immature embryos, the expression of *RAmy3D* is markedly induced by the deprivation of carbohydrates (13). In transgenic rice cell line, a *RAmy3D* promoter/GUS gene fusion as the endogenous *RAmy3D* is metabolically regulated (14). More recently, in addition, DNA-binding factors, which specifically interact with the GCCG G/C CG motifs in the promoter region of the sugar-regulated *RAmy3D*, have been initially identified (15).

Protein phosphorylation is a major regulatory mechanism of signal transduction pathways that control biological processes. In this study, to investigate whether phosphorylation events are involved in the intracellular pathways mediating α-amylase gene expression, we examined the effects of okadaic acid (OA), an inhibitor of protein phosphatases 1 and 2A (16-21), on the expression of α-amylase gene in suspension-cultured rice cells. Surprisingly, we found that OA strikingly stimulated the expression of one α-amylase gene, *αAmy3* (22), which is identical to *RAmy3D*. This increase in *αAmy3* mRNA accumulation was mainly caused by enhanced gene transcription. By using OA and specific protein kinase inhibitors or activators, we showed that protein phosphorylation is essential for regulating *αAmy3* expression and that a specific protein kinase may be involved in this signaling pathway.

MATERIALS AND METHODS

Reagents: Okadaic acid and calyculin A were purchased from GIBCO BRL and dissolved in 10% DMSO. Bt_2cAMP and 5'-AMP were purchased from Boehringer Mannheim. Actinomycin D, anisomycin, H7, H8, and W7 were obtained from Sigma.

Plant material: Rice (*Oryza sativa* L. cv Tainan 5) suspension-cultured cells were cultivated in liquid Murashige and Skoog (MS) medium (23) containing 3% sucrose and 10 μM 2,4-dichlorophenoxyacetic acid. The suspension culture was incubated at 27°C in dark and shaken at 120 rpm. The cells were subcultured every 7 days. Logarithmically growing cells (5 days after subculture) (13) were treated with reagents for assay. The collected cells were quick-frozen in liquid N_2 and stored at -70°C until use.

Southern Blot Analysis: The *αAmy* cDNA clones *αAmy3-C*, *αAmy6-C*, *αAmy7-C*, *αAmy8-C*, and *αAmy10-C* obtained from Dr Su-May Yu (22) were digested with *Eco*RI, and then electrophoresed on a 1.2% agarose gel. After staining with ethidium bromide, the gel was transferred onto a GeneScreen nylon membrane. The membrane was hybridized with the ^{32}P-labeled *αAmy3* gene-specific probe, washed as the procedures of Sambrook et al (24), and then exposed to X-ray film with intensifying screens at -70°C. The *αAmy3*

gene-specific probe was synthesized by using the PCR-amplified fragments as templates and labeled with [α-^{32}P]dCTP by the random-priming method (25).

Polymerase Chain Reaction: Two primers for PCR were chosen from the 3'-coding region and a region of the 3'-nontranslated end of *αAmy3-C*. 5'-CTCGAGGTCCCGGCGGGGCGGCACCT-3' for the 5'-primer and 5'-AAGCTTACATTATATATTGCACCAA-3' for the 3'-primer were designed according to the published sequence (8). PCR conditions were as follows: 30 sec at 94°C for DNA denaturation, 50 sec at 55°C for primer annealing, and 50 sec at 72°C for primer extension, all for 30 cycles. The amplified products (288 bp) were separated by electrophoresis in a 2% agarose gel, recovered by electroelution, and then subcloned into the plasmid vector pBluescript using the Xho I and Hind III sites.

Preparation of RNA and Northern Blot Analysis: Total RNA was extracted from cultured cells according to the previously described method (26). RNA gel blot analysis was performed according to the method of Thomas (27). The α-amylase and actin cDNA used as probes were labeled with [α-^{32}P]dCTP using the random-priming method (25) or [α-^{32}P]UTP by *in vitro* transcription (28) with T3 RNA polymerase.

Nuclear Run-on Analysis of Transcription: Nuclei were isolated from DMSO-treated (control) or OA-treated suspension-cultured rice cells following the procedures reported previously (29). Nuclei in glycerol storage buffer pH 7.8 (containing 50mM Tri-HCl, 5mM $MgCl_2$, 14mM β-mercaptoethanol, and 20% glycerol) were frozen at -70°C. The *in vitro* transcription reactions were performed as described in (30). After transcription, the reaction mixture was digested with DNase I and proteinase K, treated with phenol/$CHCl_3$, and then precipitated with ethanol containing NH_4OAc. The radioactivity of the *in vitro* transcribed RNA was determined, and equal amounts of radioactive RNA was used to hybridize to blots containing immobilized specific plasmids (5μg), previously applied to a nylon membrane by a slot blot apparatus. The nylon membrane was washed using the same conditions as for Northern blot analysis.

RESULTS

Induction of αAmy3 mRNA by okadaic acid

To analyze the effects of protein phosphorylation on α-amylase gene expression, we treated suspension-cultured rice cells with 500 nM of okadaic acid (OA). Cells were harvested 0.5 h or 1 h later. Control cells were treated with the same volume of DMSO for 1 h, since OA was initially dissolved in 10% DMSO. RNA isolated from these cells were subjected to Northern blot analysis by using *αAmy8-C* (22) as a probe to examine total expression of the α-amylase multigene family. The results in figure 1A showed that OA rapidly caused the accumulation of α-amylase mRNAs. To investigate further which α-amylase gene was induced by OA, we used the *αAmy* gene-specific probes (*αAmy6-C-3'*, *αAmy7-C-3'*, *αAmy8-C-3'*, and *αAmy10-C-3'*) (22) to hybridize the same blot. The *αAmy3* gene-specific probe designed as described under "materials and methods" was also used. Figure 1B shows that the *αAmy3* gene-specific probe only hybridizes to *αAmy3-C*, indicating that this probe is specific to *αAmy3*. To our surprise, only *αAmy3* was strongly induced by OA as shown in figure 1C. No appreciable change in rice actin mRNA was observed during the experiment.

Dose-response and time course of αAmy3 induction by okadaic acid

To understand the effects of OA on *αAmy3* expression, we treated cultured rice cells with different concentrations of this protein phosphatase inhibitor. Cells were collected 1 h later and *αAmy3* mRNA levels were analyzed by Northern hybridization with the

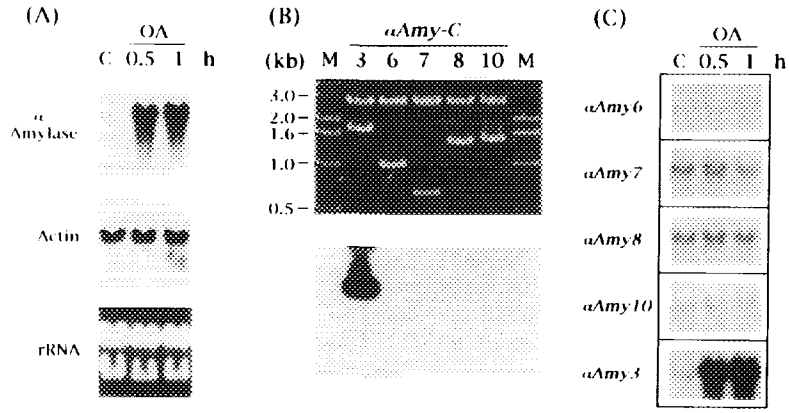

Figure 1. **(A)** Accumulation of α-amylase mRNAs after treatment of cultured rice cells with okadaic acid (OA). Cells were treated with 500 nM OA for the indicated times. The cells were also incubated with the same volume of 10% DMSO without OA as a control (C). 10 μg of total RNA was applied to each lane and subjected to Northern blot analysis by using an *αAmy8-C* cDNA probe (1.4-kb EcoRI fragment) (22). The same blot was stripped and rehybridized with a probe for actin gene to normalize the amount of RNA loaded. Ethidium bromide staining of rRNA was also shown down below. **(B)** Southern blot analysis for demonstrating the specificity of the *αAmy3* gene-specific probe. This experiment was performed as described in Materials and Methods. The 1-kb ladder (M) as molecular weight standards is shown on the left. **(C)** Accumulation of *αAmy3* mRNA induced by OA. The blots as (A) were prepared in five duplicates and hybridized with the *αAmy* gene-specific probes (*αAmy6-C-3'*, *αAmy7-C-3'*, *αAmy8-C-3'*, and *αAmy10-C-3'*) (22) as well as the *αAmy3* gene-specific probe.

αAmy3 gene-specific probe. Figure 2A showed that the increase in the accumulation of *αAmy3* mRNA correlates with the increase in OA concentration. 400 nM OA gave rise to the strongest response and induced *αAmy3* mRNA levels approximately 30~40-fold. The time course of *αAmy3* mRNA accumulation after treatment of cells with OA was also

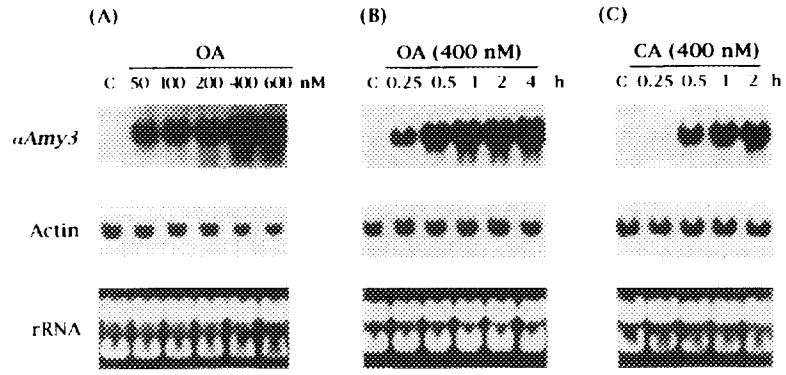

Figure 2. **(A)** Dose dependence and **(B)** time course of *αAmy3* mRNA accumulation induced by OA. Cultured rice cells were treated with the indicated concentrations for 1 h (A) or were incubated with 400 nM OA for the indicated times (B). **(C)** Accumulation of *αAmy3* mRNA induced by calyculin A (CA). Cells were treated with 400 nM calyculin A for the indicated times. Northern hybridizations were performed by using the *αAmy3* gene-specific probe or an actin probe.

examined. Under 400nM OA, this induction reached the plateau level after about 1 h of treatment, and the concentration of *αAmy3* mRNA was sustained at these levels for up to 4 h (Fig. 2B). The induction of *αAmy3* mRNA by another potent protein phosphatase inhibitor calyculin A (31), which is structurally unrelated to OA, was also studied. The expression of *αAmy3* was also strongly induced by calyculin A (Fig. 2C), but for cultured rice cells, OA was a better stimulant than calyculin A in increasing *αAmy3* mRNA accumulation (Fig. 2B vs 2C). These results showed the protein phosphatase 1 and 2A inhibitors OA and calyculin A enhance *αAmy3* expression although to different degrees.

Okadaic acid activates the transcription of αAmy3

Since OA induced *αAmy3* mRNA accumulation, we wanted to know whether OA affects the transcription rate of *αAmy3*. Cultured rice cells were treated with 400nM OA for 1 h, then nuclei were harvested and subjected to nuclear run-on transcription analysis. As shown in figure 3, OA enhanced the apparent transcriptional activity of the *αAmy3* promoter approximately ten-fold. No signal was observed with the control plasmid probe. The transcription rate of the actin gene as the control gene was not changed appreciably by OA. These results indicate that OA activates the transcription of *αAmy3* in cultured rice cells.

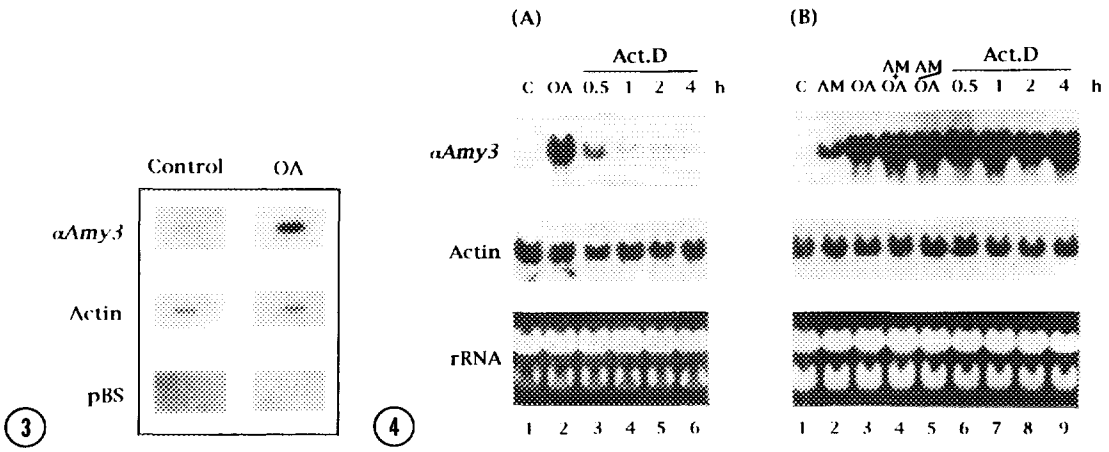

Figure 3. Nuclear run-on assay for *αAmy3* gene transcription. Nuclei were isolated from DMSO- (control) and OA- (400 nM) treated rice cells and run-on assay was performed according to the methods as described in Materials and Methods. The nylon membrane blotted with 5 μg of plasmids indicated was hybridized with the radiolabeled nascent mRNA. pBluescript (pBS) was used as a control. The intensity of ^{32}P-labeled *αAmy3* mRNA was quantitated by densitometry and normalized to that of actin mRNA.

Figure 4. (A) Effect of OA on the stability of *αAmy3* mRNA. Cells were treated 400 nM OA for 1 h, and actinomycin D (Act.D) (final concentration being 80 μM) was then added. The cells were then harvested at 0.5, 1, 2, and 4 h after the addition of actinomycin D. **(B)** Effect of protein synthesis inhibitors on OA-induced *αAmy3* mRNA. Cells were treated with either 200 μM anisomycin (AM) or 400 nM okadaic acid (OA), or both (AM+OA) for 1 h. "AM/OA" indicates that cells were pretreated with anisomycin for 1 h and then stimulated with OA for another 1 h. After "AM/OA" treatment, actinomycin D was added, and the cells were then harvested 0.5, 1, 2, and 4 h later. RNA in (A) or (B) was isolated and analyzed by Northern blotting. The blots were probed with the *αAmy3* gene-specific probe or an actin probe.

Effect of protein synthesis inhibition on αAmy3 induction by okadaic acid

The induction of *αAmy3* mRNA accumulation may be a result of mRNA stabilization. To test this possibility, we also analyzed whether OA might have an effect on the stability of this mRNA. This was examined by mRNA decay assays, and the half-life of *αAmy3* mRNA in the presence of OA was measured (Fig. 4A). Following the accumulation of *αAmy3* mRNA by OA treatment for 1 h (Fig. 4A lane 2), transcription was blocked by adding actinomycin D (Fig. 4A lanes 3 to 6). Cells were harvested at the indicated time points and RNA was extracted, followed by Northern blot analysis. The results showed that when cells were treated with actinomycin D to inhibit the synthesis of the new*αAmy3* transcripts, the OA-induced *αAmy3* mRNA rapidly disappeared from the cells. The estimated half-life of *αAmy3* mRNA in the presence of OA was 15 min. Therefore, the results presented in figures 3 and 4A indicated that the high accumulation of *αAmy3* mRNA in response to OA treatment is due to an enhanced transcription rate combined with a partial stabilization of this message. To determine whether *αAmy3* induction by OA requires new protein synthesis, cultures were pretreated with the protein synthesis inhibitor anisomycin for 1 h before OA treatment. The data indicated that the induction of *αAmy3* mRNA by OA can occur even though protein synthesis is inhibited (Fig. 4B lane 5). Cells were also treated with anisomycin in the presence or absence of OA. Notably, anisomycin alone induced *αAmy3* mRNA accumulation (Fig. 4B lane 2). Simultaneous addition of OA and anisomycin superinduced *αAmy3* mRNA accumulation more than either reagent alone (Fig. 4B lane 4 vs lanes 2 and 3). We next analyzed whether protein synthesis inhibition stabilizes OA-induced *αAmy3* mRNA. After the *αAmy3* mRNA accumulation induced by anisomycin and OA, actinomycin D was added to block RNA synthesis. The results showed that OA-induced *αAmy3* mRNA was completely stabilized in the presence of anisomycin (Fig.4B lanes 5 to 9).

Effect of protein kinase inhibitors or activators on αAmy3 induction

Because OA is a protein phosphatase inhibitor, *αAmy3* expression can be regulated by specific kinases. Thus we examined the effect of OA on *αAmy3* expression in the presence of protein kinase inhibitors. The compounds H7 and H8, inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases (32), as well as the compound W7, an antagonist of calmodulin and Ca^{2+}-dependent protein kinases (33), were applied to assess which type of protein kinases are necessary for the induction of *αAmy3* mRNA accumulation by OA. The results of this experiment (Fig. 5 lanes 6 to 9) showed that these three protein kinase inhibitors have no effect on OA-induced *αAmy3* expression. These data suggest that OA-dependent *αAmy3* mRNA induction does not require protein kinase C and Ca^{2+}-dependent protein kinases. Moreover, we also treated cultured rice cells with Bt2cAMP, an activator of cAMP-dependent protein kinase, or 5'-AMP, an inducer of AMP-activated protein kinase (34). Intriguingly, *αAmy3* induction was not observed with Bt2cAMP (Fig. 5 lane 4), but did occur with 5'-AMP (Fig. 5 lanes 2 and 3). This result indicated that a homologue of the mammalian AMP-activated protein kinase may be involved in the induction of *αAmy3* expression.

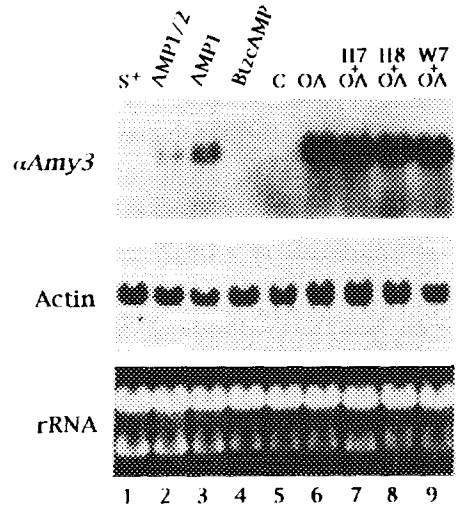

Figure 5. Effect of protein kinase inhibitors and activators on the induction of *αAmy3* mRNA. In the presence of protein kinase inhibitors H7 (100 μM), H8 (100 μM), and W7 (100 μM), cells were treated with 400 nM OA (H7+OA, H8+OA, W7+OA). Cells were also incubated with 5'-AMP (1 mM) for 0.5 h (AMP1/2) or 1 h (AMP1), or BtcAMP (1 mM) for 1 h. S^+ indicates normal suspension cells. RNA was isolated and subjected to Northern blot analysis by using the *αAmy3* gene-specific probe or an actin probe.

DISCUSSION

Okadaic acid (OA), a potent inhibitor of two types of serine/threonine phosphoprotein phosphatases 1 and 2A (PP1 and PP2A), is a powerful tumor promoter for animal cells (35). Treatment of cells with OA causes a net increase in protein phosphorylation (18) and subsequently leads to a change in the gene expression. This compound has been used extensively to investigate the mechanisms of modulation of various biological functions in higher plants (36-41). In this present study, we have shown that OA rapidly induces the expression of *αAmy3* (Fig. 1), and this induction is mainly due to the increased transcription rate (Fig. 3 and Fig. 4A). Hence we suggest that protein phosphatase (PP1 and/or PP2A) activity is involved in the signal transduction pathway that regulates the activity of *αAmy3* promoter. Under established *in vitro* assay conditions, OA inhibits PP2A at lower concentrations (~ 0.1 nM) and PP1 at higher concentrations (~ 10 nM) (19). However, we do not know which protein phosphatase is required for OA-dependent *αAmy3* induction, because the cellular concentration of OA cannot be manipulated.

The OA-induced *αAmy3* mRNA has a very short half-life (Fig. 4A), suggesting that this rapid mRNA turnover contributes little to the accumulation of *αAmy3* mRNA by OA. It has been shown that α-amylase mRNAs are rather stable (~ 12 h of half-life) in sucrose-starved cells, as well as that the half-life of α-amylase mRNAs is reduced to less than 1 h by providing sucrose (30). During the experiment, in addition, OA affected cells in the presence of sucrose. It is likely that sucrose causes OA-induced *αAmy3* mRNA to

undergo a rapid turnover in cultured rice cells. By using OA combined with protein synthesis inhibitor anisomycin, we found that the high-level accumulation of *αAmy3* mRNA induced by OA does not require newly synthesized proteins. Furthermore, in the presence of anisomycin, in contrast, the OA-induced *αAmy3* mRNA was completely stabilized (Fig. 4B). Another protein synthesis inhibitor cycloheximide has been suggested previously to cause the accumulation of α-amylase mRNAs (30). Here we also found that anisomycin induces the accumulation of *αAmy3* mRNA (Fig. 4B). The above findings suggest the possibility that a RNase which specifically degrades *αAmy3* mRNA might be present in cultured rice cells, but the synthesis of this labile RNase is probably blocked by anisomycin and subsequently the OA-induced *αAmy3* mRNA is apparently stabilized in cells provided with sucrose. It is not known whether protein synthesis inhibitors increase *αAmy3* mRNA abundance by enhancing the transcription rate, however, they strengthen the stability of this message. Studies in animal systems have shown that OA inhibits cellular protein synthesis at the translational level (42, 43), but within 15 min *αAmy3* mRNA accumulation is initially induced under 400 nM OA (Fig. 2B), suggesting that the induction of *αAmy3* expression by OA is not likely due to the effect of OA on the translation rates. Therefore, we can propose that both protein phosphatase inhibitors and protein synthesis inhibitors can induce *αAmy3* mRNA accumulation, but respectively by means of two different mechanisms. So it is reasonable to confirm that simultaneous treatment of OA and anisomycin causes a synergistic effect upon *αAmy3* expression.

As OA blocks the function of both PP1 and PP2A, the increased net activity of protein kinases in cultured rice cells leads to *αAmy3* mRNA accumulation. Experiments were performed in which protein kinase inhibitors H7, H8, and W7 in combination with OA were used to examine the effects on *αAmy3* expression. Blockage of protein kinase C and cyclic nucleotide-dependent protein kinases with H7 or H8, as well as blockage of calmodulin and Ca^{2+}-dependent protein kinases with W7 have no effect on *αAmy3* induction by OA (Fig. 5 lanes 6 to 9). Hence studies presented here indicate that the signaling pathway mediating the *αAmy3* expression involves phosphorylation events and is regulated by specific kinases, but is independent of protein kinase C and Ca^{2+}/calmodulin- or Ca^{2+}-dependent protein kinases. Furthermore, recent experiments have demonstrated that the expression of *αAmy3* (*RAmy3D*) is controlled by metabolic regulation in rice cell cultures (12-14). It is apparent that the level of ATP will markedly decline in cultured rice cells subjected to sugar starvation. Subsequently, the depletion in the concentration of ATP will lead to a concomitant increase in the level of AMP. In addition, it has been shown previously that the rise in AMP level elevated by incubation of isolated hepatocytes with fructose causes activation of the AMP-activated protein kinase (44). It has also been suggested that one function of the AMP-activated protein kinase is as a type of metabolic stress response (45). Therefore, it is plausible that AMP could be considered as a matabolic signal for modulating the activity of the AMP-activated protein kinase (46). On the basis of the above studies, we tried to test the effect of AMP on *αAmy3* expression. The induction of *αAmy3* mRNA accumulation was specifically observed with

AMP (Fig. 5 lanes 2 and 3), but did not occur with Bt2cAMP, an analogue of cAMP (Fig. 5 lane 4). Thus it is reasonable to propose that an AMP-activated protein kinase may be involved in the signal transduction pathway that regulates *αAmy3* expression. Further experiments are necessary to identify the role of AMP in metabolic regulation of *αAmy3* promoter as well as whether the AMP-activated protein kinase is essential for this signaling pathway.

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