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Superinduction of the *Em* gene in rice suspension cells in the presence of ABA and cycloheximide

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Abstract The *Em* gene in rice (*Oryzae sativa* L.) cell suspensions is strongly induced in the presence of abscisic acid (ABA) or in the presence of an osmoticum such as NaCl. Experiments were conducted to determine the impact of protein synthesis inhibitors on *Em* mRNA accumulation as part of a study to further characterize regulation of this gene in rice. When rice cell suspensions were incubated in the presence of ABA or NaCl and either 10 μM cycloheximide (resulting in a 42% inhibition of net protein synthesis) or 100 μM cycloheximide plus 10 μM chloramphenicol (93% inhibition of net protein synthesis), *Em* mRNA accumulated to a level more than twofold greater than in cell suspensions treated with either ABA or NaCl alone. Neither cycloheximide nor chloramphenicol alone or in combination induced *Em* gene expression. When rice cells were exposed to 50 μM ABA for 1 h and then transferred to medium without ABA, *Em* transcript abundance increased until 24 h and then declined. The rate of this decline was slower in the presence of the protein synthesis inhibitors. The results indicate that *de novo* protein synthesis is not required for ABA or NaCl induction of *Em* gene expression in rice suspension cells and that superinduction can occur when protein synthesis is only partially blocked in the cell population.

Key words Abscisic acid · Cycloheximide · LEA genes · Osmotic stress · Superinduction

Abbreviations *ABA* Abscisic acid · *2,4-D* 2,4-dichlorophenoxyacetic acid · *Em* early methionine-labelled polypeptide · *LEA* late embryo abundant

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Introduction

Protein synthesis inhibitors can strongly influence the abundance of certain classes of mRNAs in eukaryotic cells. The accumulation of some of these transcripts may be enhanced and prolonged in the presence of cycloheximide or anisomycin, a process called superinduction. In mammalian cells, transcripts for the proto-oncogenes c-*myc* and c-*fos* are superinduced and stabilized when induced cells are incubated in the presence of protein synthesis inhibitors (Dani et al. 1984; Greenberg et al. 1986; Makino et al. 1984; Mitchell et al. 1986). The decay of mRNAs for c-*myc* and c-*fos* is less rapid in the presence of the inhibitors, suggesting that there is a significant post-transcriptional component in the quantitative expression of these genes (Levine et al. 1986; Linial et al. 1985; Mitchell et al. 1986). The mechanisms for superinduction are thought to include a loss of transcriptional repressors and ribonucleases, a requirement for translational processing of mRNAs before they can be degraded, and, in some cases, a direct transcriptional activation by the protein synthesis inhibitors (Gay et al. 1989; Mahadevan and Edwards 1991). In the case of c-*myc*, the effect of cycloheximide on mRNA abundance is likely due to an effect on a *trans*-acting factor whose synthesis is blocked by translational inhibitors (Ross 1995). In higher plants, superinduction by protein synthesis inhibitors is relatively unexplored, but several auxin-responsive mRNAs are also induced by cycloheximide alone (Theologis et al. 1985; van der Zaal et al. 1987) and superinduced in soybean by cycloheximide in the presence of auxin (Franco et al. 1990). Factors involved in the control of mRNA stability in plants have recently been reviewed (Abler and Green 1996), and progress has been made in the identification of *cis*-acting elements and *trans*-acting factors that influence the stability of certain short-lived messages.

As part of a study on the regulation of the ABA-responsive *Em* gene in rice, we observed a superinduction of *Em* mRNA in ABA-treated rice suspension cells when cycloheximide was included in the medium during the treatment.

The *Em* gene is a member of the LEA D-19 family and encodes one of the most abundant cytosolic proteins accumulating in embryos of dry cereals (Grzelczak et al. 1982; Cuming 1984; Quatrano et al. 1993). *Em* provides a model for the study of ABA-regulated gene expression in plants (Rock and Quatrano 1995). High levels of *Em* mRNA accumulate during embryo maturation (McCarty et al. 1991; Quatrano et al. 1993) and in vegetative tissue exposed to ABA or osmotic stress (Hetherington and Quatrano 1991; Bostock and Quatrano 1992). The expression of *Em* in wheat embryos and embryogenic rice cell suspensions is transcriptionally regulated (Williamson and Quatrano 1988; Marcotte et al. 1988). Additional control at the post-transcriptional level is suggested by experiments in which the transcriptional inhibitor α-amanitin prevents the decline in *Em* mRNA levels that normally occurs when wheat embryos are excised from the seed (Williamson and Quatrano 1988). The *Em* gene and its promoter are well-characterized, and transcription factors and *cis*-regulatory elements involved in the response to ABA and VP1 have been identified (Marcotte et al.1989; Guiltinan et al.1990; Hill et al. 1996; Rock and Quatrano 1994; Nantel and Quatrano 1996; Razik and Quatrano 1997; Schultz et al. 1996; Vasil et al. 1995). However, our understanding of the network of factors which interact to contribute to the level of *Em* expression is incomplete. In this paper, we present the results of experiments that were carried out to further characterize the effect of protein synthesis inhibitors on *Em* gene expression.

Materials and methods

Rice cell suspensions and treatments

The experimental system used in this study is described in a previous article (Bostock and Quatrano 1992). Rice cell suspensions were initiated from callus and maintained on liquid N6 medium containing 2 mg l^{-1} 2,4-D and 3% (w/v) sucrose, pH 5.8, at 22 °C in the dark on a rotary shaker at 110 rpm (Marcotte et al. 1988; Chu et al. 1975). Suspensions were subcultured weekly with fresh medium, and subcultures were used for experiments 5–7 days after transfer. For experimental treatments, aliquots from ethanolic stock solutions of (+/–)2-*cis*-4-*trans*-ABA (100 μM), cycloheximide (36 μM), and chloramphenicol (31 μM) were added to the cultures. NaCl was from a 5 M aqueous stock solution. Cultures were incubated in the presence of the protein synthesis inhibitors for 1.5 (Fig. 1) or 6 h (as indicated in the text) prior to the addition of ABA or NaCl treatments. In some experiments, cultures were incubated for 24 h and then harvested for RNA extraction. To compare the rates of decay of *Em* mRNA: 10-ml cultures were treated with protein synthesis inhibitors for 6 h, pulsed with 50 μM ABA for 1 h, washed 3 times with fresh N6 medium, and then incubated in 10 ml of fresh medium containing protein synthesis inhibitors until extraction. The control treatment excluded protein synthesis inhibitors both before and after ABA treatment. All chemicals were purchased from Sigma Chemical, St. Louis, Mo.

RNA extraction and gel blot analyses of *Em* gene expression

Treated cultures were sampled by removing the medium, transferring the cells to a polypropylene tube, and then immediately freezing the cells in liquid N_2. The frozen cells were stored at –80 °C until extraction. A modification of the rapid RNA extraction procedure of Chomczynski and Sacchi (1987) was used throughout this study and is described in a previous paper (Bostock and Quatrano 1992). Total RNA (10 μg per lane) was separated on 1.2% agarose gels containing formaldehyde as described in Ausubel et al. 1987. After electrophoresis, the RNA was transferred to nylon membranes (Hybond-N, Amersham) using 10×SSC and UV-crosslinked to the membrane. The RNA was hybridized with a random-primed, [^{32}P]-dCTP-labelled fragment from p1015 which has a 608-bp *Pst*I insert containing the entire coding region, 5′ and 3′ flanks, and a poly-A tail of the wheat *Em* gene (Litts et al. 1987). The conditions of hybridization were those described previously (Bostock and Quatrano 1992). The radioactivity on the washed membranes was counted directly using a radioanalytic imaging system (Ambis Systems, San Diego, Calif.) or indirectly by scanning densitometry (Molecular Dynamics, Sunnyvale, Calif.) of autoradiograms. In addition to the visual estimation of gel lane loading by the comparison of ethidium stained ribosomal RNA bands, some blots were hybridized with a probe prepared from pACT2-3A1, which contains a 1.65 kbp rice actin cDNA (gift of R. B. Meagher), to assess any treatment effects on a constitutively expressed gene as an internal standard.

Determination of net protein synthesis

Protein synthesis rates were estimated by the incorporation of [^{35}S]-L-methionine into protein per hour per gram fresh weight of rice suspension cells by a modification of the method described by Stermer and Bostock (1989). A 10-μl drop containing 4.2×10^5 Bg [^{35} S]-L-methionine (Amersham, 4.3×10^{10} Bg mmole^{-1}) and 11.1 mM L-methionine (Sigma) was added to each culture. Cultures were incubated for 2 h with shaking at 22 °C, the cells were collected with a spatula and blotted on filter paper, fresh weights were determined, and the tissue transferred to a 1.5-ml microfuge tube containing 700 μl of protein extraction buffer. The buffer consisted of 50 mM Tris-HCl, pH 8.3, 2% (w/v) SDS, 5 M urea, and 2% 2-mercaptoethanol. The cells were homogenized in the microfuge tube with a pellet pestle, vortexed, and placed at room temperature overnight. The vial was then vortexed and centrifuged for 5 min at 10 000 *g*. Four hundred microliters of the supernatant were transferred to a test tube and the proteins precipitated by the addition of 11.1% TCA. The samples were kept on ice for 1 h and the precipitates collected on Whatman GF/A filter disks (2.4 cm) by vacuum filtration. The filters were air-dried and washed by a modification of the procedure described by Sutton and Shaw (1986). Three to six filters were immersed with shaking for 10 min sequentially in 50 ml of each of the following solutions: ice cold 10% TCA containing 1 mg ml^{-1} L-methionine, 10% TCA at 95 °C, 10% TCA at room temperature, and 95% ethanol. The filters were air-dried and the radioactivity determined by liquid scintillation.

Results and discussion

Treatment of rice cell suspensions with optimal concentrations of ABA (200 μM) or NaCl (0.4 M) resulted in a strong induction of *Em* mRNA accumulation within 24 h of treatment as previously reported (Bostock and Quatrano 1992). *Em* transcripts were not detected in nontreated control cultures. Treatment of rice cells with cycloheximide (10 μM) for 1.5 h prior to the addition of ABA or NaCl resulted in a doubling of the ABA- or NaCl-induced *Em* mRNA accumulation when compared to ABA or NaCl alone (Fig. 1A, B). At this concentration, cycloheximide resulted in a 42% inhibition in net protein synthesis at the time of application of the inducing treatment. Treatment of the cultures with a combination of cycloheximide (100 μM) and chloramphenicol (10 μM) for 6 h prior to the

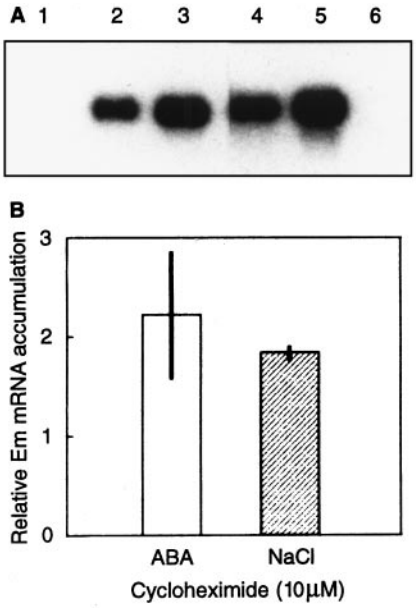

Fig. 1 **A** Autoradiogram of an RNA gel blot showing ABA and NaCl induction of *Em* mRNA and superinduction in the presence of cycloheximide in rice suspension culture cells. Total RNA (10 μg per lane) was hybridized with a [^{32}P]-labelled probe from p1015 which detects a transcript of approximately 820 bp. The cultures were either untreated (*lane 1*) or treated with 200 μM ABA (*lane 2*), 200 μM ABA and 10 μM cycloheximide (*lane 3*), 0.4 M NaCl (*lane 4*), 0.4 M NaCl and 10 μM cycloheximide (*lane 5*), or 10 μM cycloheximide (*lane 6*). **B** Superinduction of *Em* mRNA in the presence of 10 μM cycloheximide. Values are expressed relative to the corresponding control (a value of 1.0) for each treatment (200 μM ABA or 0.4 M NaCl). Cells were exposed to 10 μM cycloheximide for 1.5 h prior to ABA or NaCl addition, a treatment that results in a 42% inhibition of net protein synthesis. Values are the means and SE from at least two separate experiments for each treatment

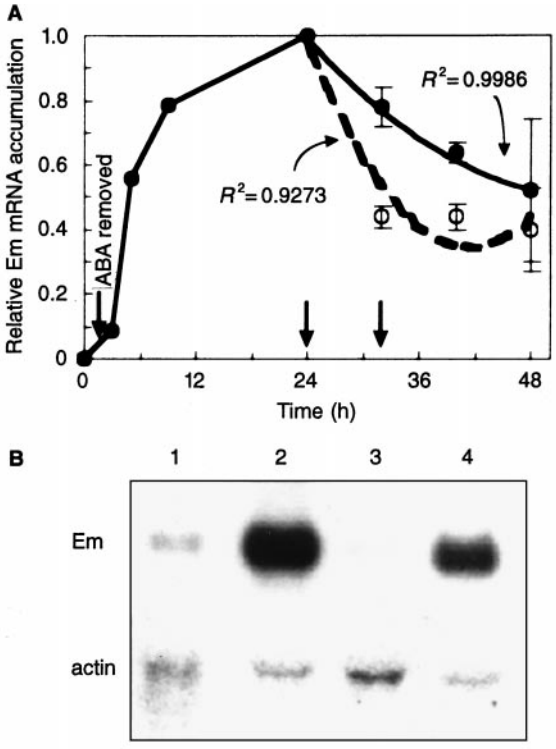

Fig. 2 **A** Time course of induction and decline of *Em* mRNA levels following a 1 h exposure of rice suspension culture cells to 50 μM ABA in the presence or absence of protein synthesis inhibitors. Values are expressed relative to the maximum abundance within each treatment and are the means and SE from two experiments. ● With 100 μM cycloheximide and 10 μM chloramphenicol, ○ without inhibitors, R^2 values for the second-order polynomial regression trendlines are indicated. **B** RNA gel blot showing the abundance of *Em* mRNA and actin mRNA in rice suspension culture cells exposed to 50 μM ABA in the presence or absence of protein synthesis inhibitors at 24 h after initiation of the experiment (*lanes 1* and *2* – and + inhibitors, respectively) or at 32 h after initiation of the experiment (*lanes 3* and *4* – and + inhibitors, respectively)

introduction of ABA or NaCl inhibited net protein synthesis by 93% and resulted in a 2.5-fold superinduction of the ABA-induced *Em* mRNA levels (data not shown). Chloramphenicol alone did not induce Em mRNA accumulation. The effect of the inhibitors on the NaCl-induced transcript levels was somewhat variable and ranged from no effect to more than a twofold enhancement of that in cultures induced by NaCl alone. Neither cycloheximide nor chloramphenicol alone or in combination induced the accumulation of transcripts for *Em*. Together, these results indicate that the ABA or NaCl induction of *Em* gene expression in rice cell suspensions can occur when *de novo* protein synthesis is almost completely inhibited and that superinduction can occur when protein synthesis is only partially blocked in the cell population. Because superinduction could be observed with only cycloheximide as the inhibitor (Fig. 1A, B), the effect is probably mediated primarily through the 80S ribosomal translational apparatus.

To determine if a decline in *Em* mRNA abundance occurs after induction with and subsequent removal from ABA and if this decline is affected by protein synthesis, we first established conditions under which we could observe *Em* mRNA induction and decay. Preliminary experiments indicated that *Em* mRNA levels remained elevated over several days with the continued presence of ABA in the rice cell suspensions. By pulsing the rice cultures with 50 μM ABA for 1 h and then washing the cells with fresh, ABA-free medium, we observed a strong induction of *Em* mRNA, with transcript levels increasing to a maximum by 24 h, followed by a significant decline during the subsequent 24-h period (Fig. 2). Under these inductive conditions, *Em* mRNA was superinduced in the presence of protein synthesis inhibitors but the decline in transcript levels was considerably less rapid (50% of maximum levels attained 24 h later) than in the control cell suspensions (50% of maximum levels about 7 h later; Fig. 2A). In addition, the abundance of actin mRNA, although low, was similar in RNA extracts from inhibitor-treated and untreated cell cultures (Fig. 2B), and the small variation among samples observed cannot explain the pronounced treatment effects on *Em* message levels. These results affirm that *Em* transcripts represent a substantially greater proportion of the RNA population recovered from the inhibitor-treated cell cultures than from the untreated cell cultures.

Our results could be explained by the participation of a factor(s), dependent on protein synthesis, involved in the transcriptional repression of *Em* expression or in the degradation of *Em* mRNA, or both. Because only net accumulation of *Em* mRNA was measured, we could not distinguish between transcript synthesis and degradation and, therefore, did not establish a true "half-life" for *Em* mRNA. Sequence comparison of the *Em* cDNA with *cis*-acting determinants conferring mRNA instability in other plant genes, such as the *SAUR-AC1* 3′ end, DST consensus, and AUUUA repeats (Abler and Green 1996), did not reveal any regions of significant similarity. This is not unexpected since the *Em* message would not be considered an unstable transcript (half-life of less than an hour) typical of those in which these sequences occur.

In an earlier study (Bostock and Quatrano 1992), we showed that response-saturating concentrations of ABA and NaCl, when added together to rice cell suspensions, were strongly synergistic in their induction of *Em* mRNA and resulted in a doubling of transcript levels over those in cells treated with either inducer alone. Subsequently, it was shown that other ions, notably calcium and lanthanum, can act synergistically with ABA to induce *Em* gene expression (Rock and Quatrano 1996). Thus, NaCl and other ions appear to change the sensitivity of the cells to ABA. Our previous study further suggested that NaCl operated, in part, through an ABA-dependent pathway and, in part, through an ABA-independent pathway to induce *Em* mRNA accumulation. Although NaCl can inhibit protein synthesis in plants (Hurkman and Tanaka 1987), our results indicate *Em* induction by NaCl involves more than this since the inhibition of protein synthesis alone is insufficient to trigger expression. Our results also indicate that all the proteins necessary for the induction of *Em* by ABA or NaCl are constitutively present in rice suspension cells. *Em* is therefore similar to the ABA-regulated *Rab* gene in rice, which also responds to ABA in the presence of protein synthesis inhibitors (Mundy and Chua 1989). However, *Rab* mRNA is not superinduced in the presence of ABA and cycloheximide.

To our knowledge this is the first report of the superinduction of an ABA-regulated gene by protein synthesis inhibitors, and our investigation demonstrated that the phenomenon of superinduction is not limited to auxin responsive genes in higher plants. However, unlike the auxin responsive genes which are transiently induced by cycloheximide (Franco et al. 1990), *Em* was not induced by cycloheximide alone. With respect to the models for superinduction discussed above, our findings would appear to discount a role for transcriptional activation by cycloheximide and favor an important role for proteins that influence the stability and turnover of *Em* transcripts that are already induced by effectors such as ABA and NaCl. Transcription run-on experiments and more quantitative decay kinetic analyses would help resolve the mechanistic basis for our observations. Collectively, our results and those of another study (Williamson and Quatrano 1988) suggest an important post-transcriptional effect on the quantitative expression of *Em* in cereals, and they should lead to experiments to identify the specific mechanisms responsible for the cycloheximide effect on *Em* gene expression.

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