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A TGACGT Motif in the 5′-Upstream Region of α-Amylase Gene from *Vigna mungo* is a *cis*-Element for Expression in Cotyledons of Germinated Seeds

Daisuke Yamauchi [1]

Department of Biological Sciences, Tokyo Metropolitan University, Minami-ohsawa 1-1, Hachioji, Tokyo, 192-0397 Japan

α-Amylase is expressed at high levels in cotyledons of germinated seeds of *Vigna mungo*. The mRNA for α-amylase appeared in cotyledons of the seeds at 1 d after imbibition started (DAI). Two TGACGT motifs at –445 and at –125 in the promoter region of the gene interacted with nuclear proteins from cotyledons of dry seeds and the activities were detected until 3 DAI. A transient assay with particle bombardment showed that the downstream region from –135 in the promoter was required for high level expression in the cotyledons and the activity was reduced by mutation of the TGACGT motif at –125. The activities to bind the TGACGT motifs were detected in the axes of the seeds at 1 DAI but disappeared at 4 DAI, although the mRNA for α-amylase in the axes appeared at 4 DAI and increased in level by 6 DAI. A transient assay experiment showed that a positive regulatory element for the expression in the axes was located in the region from –630 to –453. These results indicated that the TGACGT motif at –125 was required for high level expression of the gene in the cotyledons of the germinated seeds.

Key words: α-Amylase (EC 3.2.1.1) — Seed germination — Transcription factor — *Vigna mungo*.

Abbreviations: CaMV35S, cauliflower mosaic virus 35S RNA; DAI, day after imbibition started; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; LUC, luciferase.

Introduction

Storage reserves in seeds are degraded to support the growth of seedlings. α-Amylase is involved in the degradation of starch (Bewley and Black 1994). In cereals, the expression of the genes for the enzyme is induced by gibberellins. A *cis*-acting element for the gibberellin-responsive gene expression has been identified in the promoter regions of the genes for α-amylase (Skriver et al. 1991, Gubler and Jacobsen 1992, Lanahan et al. 1992). The sequence for the gibberellin-responsive gene expression in the promoter region of the α-amylase gene interacted with GAMyb, a protein containing the Myb DNA-binding domain, which is a *trans*-acting element for the expression of the α-amylase gene (Gubler et al. 1995). ABA represses the expression of the gene for α-amylase and the protein kinase, PKABA1, mediates this repression (Gómez-Cadenas et al. 1999). A transcription factor, VIVIPAROUS1 (VP1), also suppresses the gibberellin-induced expression of the α-amylase gene, but VP1 and ABA inhibit the gene expression independently (Hoecker et al. 1995). Little is known, however, about the regulation of the gene expression of the enzyme in dicot seeds. The gene for α-amylase has been isolated from *Vigna mungo* (Takeuchi et al. 1993). A genomic Southern blot analysis indicated that the enzyme is encoded by a single copy gene (Yamauchi et al. 1994). In the 5′-upstream region, two motifs, TGACGT and CATGCAT, were found (Yamauchi et al. 1994). The TGACGT sequence is known to interact with basic leucine zipper bZIP DNA binding proteins (Katagiri et al. 1989, Tabata et al. 1989). The other motif, CATGCAT, is involved in the ABA-responsive gene expression (Hattori et al. 1992) and the transcription factor VP1 interacted with the sequence (Suzuki et al. 1997). However, it is unclear whether the CATGCAT motifs in the 5′-upstream region of the α-amylase of *V. mungo* are involved in the expression in response to ABA or VP1-like transcription factors, because the synthesis of α-amylase in the cotyledons is repressed by the exogenous application of ABA (Taneyama et al. 1995).

To determine whether the TGACGT motifs in the upstream region of the gene for α-amylase from *V. mungo* are involved in the gene expression, I examined the binding of nuclear proteins to the motifs. Transient assay experiments indicated that the TGACGT motif at –125 is required for the expression of the α-amylase gene in cotyledons of the germinated seeds.

Materials and Methods

Plant materials

Seeds of *V. mungo* were purchased from Takii Seed (Kyoto, Japan). The seeds were germinated as described previously (Mitsuhashi and Minamikawa 1989). The plants of *V. mungo* were grown at the experimental farm of the Department of Biological Science, Tokyo Metropolitan University. The seeds were collected from fruits 20 d after flowering and stored at –80°C until use.

RNA blot analysis

The total RNA fraction was prepared by the phenol extraction method as described by Mitsuhashi and Minamikawa (1989). RNA blot analysis was carried out as described previously (Tanaka et al. 1993). The full-length cDNA for α-amylase from *V. mungo* (Yamauchi and Mianamikawa 1990) was used as the probe. Autoradiography was

[1] Corresponding author: E-mail: yamauchi-daisuke@c.metro-u.ac.jp; Fax: +81-426-77-2559.

Table 1 Nucleotide sequences of DNAs for probes and competitors

Gene product	Probe/Competitor		Sequence	
Amylase	A1	–135	atcttgaTGACGTAaatttcac	–114
	$A1^m$	–135	atcttgaTGAC*ca*Aaatttcac	–114
	A2	–453	gatgaactACGTCAacatcttc	–432
	$A2^m$	–453	gatgaactAC*ca*CAacatcttc	–432
SH-EP	E1	–430	caaaacagGACGTaaaccatgtt	–409

Sequences corresponding to the TGACGT motif are indicated in capital letters. Mutated sequences are shown in italics.

carried out with X-ray film (X-Omat AR, Eastman Kodak Company, Rochester, U.S.A.).

Electrophoretic mobility shift assay (EMSA)

Preparation of crude nuclear extract was performed as described by Mikami et al. (1987), except that the frozen powder from the cotyledons or axes was homogenized in 50 mM Tris-HCl (pH. 7.9), 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1.6 mM salicylhydroxamic acid, 1 μg ml^{-1} *t*-butylated hydroxytoluene, 5 mM dithiothreitol by an ultradisperser (Janke & Kunkel, Staufen, Germany) at high speed for 1 min. The protein concentration was determined by the method of Bradford (1976). The DNA probe was labeled with polynucleotide kinase. The EMSA reaction was carried out as described by Mikami et al. (1987), except that the crude extract was incubated with 10 fmol ^{32}P-labeled DNA (about 40,000 cpm) and 1 μg poly dI-dC/poly dI-dC on ice for 30 min. The assay mixture was separated by 4% PAGE according to Singh et al. (1986). After electrophoresis, the gel was dried on a filter paper and autoradiographed.

Construction of reporters

An *Nhe*I site was created at +29 of the genomic clone of the α-amylase gene, pVMAMY1A (Takeuchi et al. 1993), by PCR with a reverse primer for sequencing and AmyP-1 primer (5′-AGAATCGCTAGCTTTGAT). After digestion of the amplified fragment with *Hin*dIII and *Nhe*I, the fragment carrying the sequence from –639 to +29 of the promoter region was inserted between the *Hin*dIII and *Xba*I sites of pBI221 to replace the cauliflower mosaic virus 35S RNA (CaMV35S) promoter (Jefferson et al. 1987). The resulting plasmid was designated pAmy-639. The oligonucleotide probes A1, A2 and $A1^m$ (Table 1) were used for amplification of the promoter region of the α-amylase gene by PCR with the AmyP-1 primer. Each fragment was cloned into TA vector (Clontech, Palo Alto, U.S.A.). These fragments were cut with *Hin*dIII and *Nhe*I and were inserted into the *Hin*dIII and *Xba*I sites of pBI221, and the resulting plasmids were named pAmy-453, pAmy-135 and pAmy-135m, respectively. A *Hin*dIII site at –103 or –44 was created by PCR with the AmyP-1 primer. The amplified fragment was inserted between the *Hin*dIII and *Xba*I sites of pBI221 to obtain pAmy-103 and pAmy-44, respectively.

Particle bombardment

Particle bombardment was carried out as described previously (Yamauchi 1997), except that gold particles coated with plasmids were used to bombard the cotyledons of the germinated seeds of *V. mungo* at 1 d after imbibition started (DAI). After the particle bombardment, the seedlings were incubated on wet filter paper at 27°C in darkness for 24 h. For bombardment of the embryonic axes, seedlings at 3 DAI were used. After the bombardment, the axes were incubated in a Petri dish containing 5 mM sodium phosphate buffer (pH7.2) at 27°C in darkness for 24 h. An enzyme assay was carried out as described previously (Yamamoto et al. 1995).

Results

Expression of α-amylase in seedlings

Starch is stored in the cotyledons of *V. mungo* seeds and is degraded by α-amylase (Minamikawa et al. 1992). The expression of α-amylase in the germinated cotyledons is regulated at the mRNA level (Yamauchi et al. 1994). The mRNA was detected in the cotyledons at 1 DAI and then its level increased at 2 DAI (Fig. 1). α-Amylase is synthesized in the axes of seedlings but its activity is lower than in germinated cotyledons (Minamikawa et al. 1992). The mRNA for α-amylase was detected in the axes of the seedlings at 4 DAI and its level increased until 6 DAI (Fig. 1). However, the level in the axes at

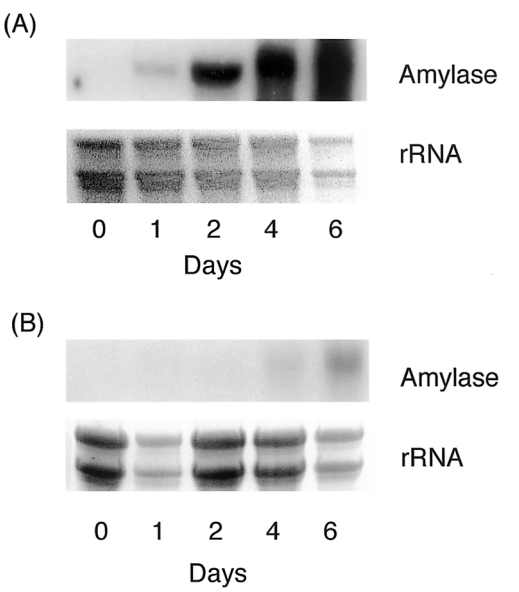

Fig. 1 Changes with time in the α-amylase mRNA level in the cotyledons (A) or axes (B) during the germination of seeds of *V. mungo*. Total RNAs from the cotyledons (10 μg per lane) or axes (40 μg per lane) were transferred onto a nylon membrane after separation by a 1.4% agarose gel. The α-amylase mRNA was probed by the ^{32}P-labeled α-amylase cDNA (Amylase). The membrane was exposed to X-ray films for 1 d (A) or 1 week (B). Gels stained with 0.04% methylene blue in 0.5 M sodium acetate buffer, pH5.2 are shown at the bottom of each blot (rRNA).

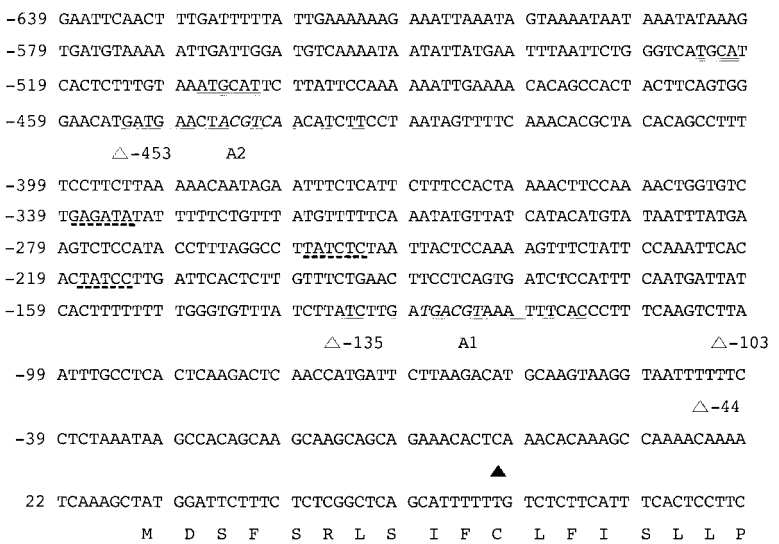

Fig. 2 Nucleotide sequence of the promoter region of the α-amylase gene from *V. mungo*. The lines indicate the sequences of synthetic oligonucleotides used for EMSA. The open and closed triangles indicate the end points of 5′ deletion mutants and the site of initiation of transcription, respectively. The dotted lines and double underlines denote the amylase element-related sequences and the repeat sequences of ATGCAT, respectively. The numbers indicate the distance from the site of initiation of transcription.

6 DAI was lower than that in the cotyledons at 1 DAI, because a long exposure to X-ray film was necessary to detect the mRNA in the axes.

Nuclear proteins interacting with TGACGT motifs

Two TGACGT motifs were found in the promoter region of the α-amylase from *V. mungo* as shown in Fig. 2. I postulated that these motifs are involved in the gene expression, because they are known to interact with nuclear proteins (Katagiri et al. 1989). To detect proteins binding to the motifs, I used two probes as described in Table 1. The crude nuclear extracts prepared from the cotyledons of immature and germinated seeds of *V. mungo*, were incubated in a tube on ice, because nuclease in the extracts degraded the probe DNAs during incubation at room temperature. No binding activity of the extract from the immature seeds for either probe could be detected (Fig. 3). Nuclear proteins from the dry seeds interacted with probe A1 and formed two bands. The intensity of these two bands decreased at 1 DAI. The band with low mobility vanished on day 2 but the band with high mobility could be detected until 3 DAI. The change in activity to bind probe A2 was similar to that to bind A1, but the level of activity was lower than for A1.

A competition assay was carried out to determine the specificity of the protein binding to the sequence (Fig. 4). Each probe was mutated by changing the core sequence from ACGT to ACCA (Table 1). The mutated oligonucleotide is indicated by the superscript "m". The ACGT motifs were found in the promoter region of the gene for a cysteine-endopeptidase, SH-EP (Yamauchi et al. 1994). Oligonucleotide E1 was chosen as shown in Table 1, because this sequence, GGACGT, is similar to the two motifs in the α-amylase promoter. The addition of A1 inhibited the formation of the complexes within A1. The addition of A2 inhibited the formation of the protein–DNA complex weakly. The additions of E1 and $A1^m$ did not affect the formation. The complexes within A2 did not form on addi-

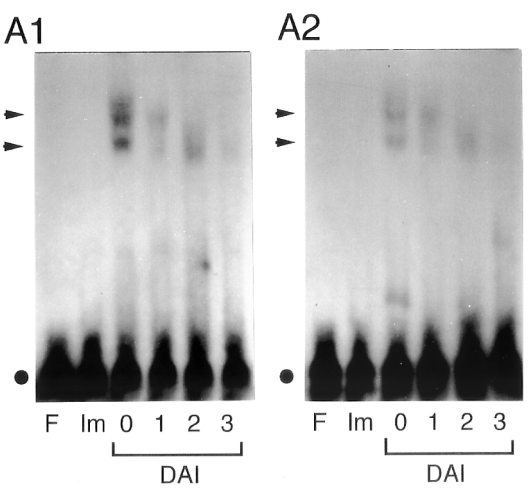

Fig. 3 Analysis of the binding activities of nuclear extracts from cotyledons at different stages for the oligonucleotide probes. Oligonucleotides, A1 and A2 were used as probes for EMSA. The reaction mixture for EMSA contained 400 μg of protein of the crude nuclear extracts. DAI, days after imbibition started. Lanes: F, no extracts; Im, extracts from immature seeds 20 d after flowering; 0, dry seeds; 1, 1 DAI seeds; 2, 2 DAI seeds; 3, 3 DAI seeds. The arrows and closed circles indicate the major specific DNA-protein complexes and free probes, respectively.

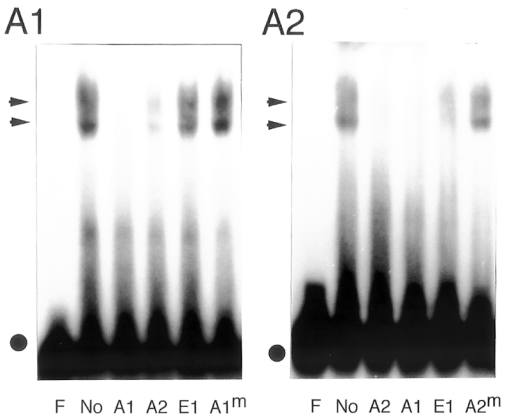

Fig. 4 Competition assays with oligonucleotides. Oligonucleotides A1 and A2 were used as probes for EMSA. The reaction mixture contained the crude nuclear extracts from dry seeds (400 μg of protein) and a 100-fold molar excess of an oligonucleotide as a competitor. Lanes: F, no extracts; No, reactions without a competitor; A1, plus competitor A1; A2, plus competitor A2; E1, plus competitor E1; $A1^m$, plus competitor $A1^m$; $A2^m$, plus competitor $A2^m$. The arrows and closed circles indicate the major specific DNA–protein complexes and free probes, respectively.

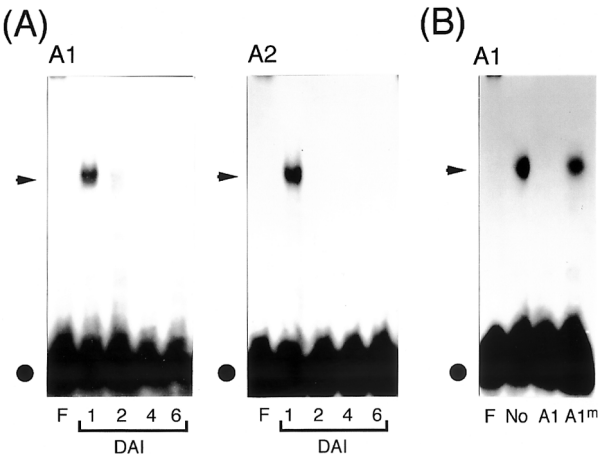

Fig. 5 Analysis of the binding activities of the nuclear extracts from the axes. (A) Nuclear extract from the axes at different stages. Oligonucleotides, A1 and A2, were used as probes for EMSA. The reaction mixture for EMSA contained the crude nuclear extracts (40 μg of protein). DAI, days after imbibition started. Lanes: F, no extracts; 1, 1 DAI seeds; 2, 2 DAI seeds; 4, 4 DAI seeds; 6, 6 DAI seeds. The arrows and closed circles indicate the specific DNA–protein complexes and free probes, respectively. (B) Competition assay with oligonucleotides. The oligonucleotide A1 was used as a probe for EMSA. The reaction mixture contained 40 μg of protein of the crude nuclear extracts from the 1 DAI axes and a 100-fold molar excess of an oligonucleotide as a competitor. Lanes: F, no extract; No, reaction without a competitor; A1, plus competitor A1; $A1^m$, plus competitor $A1^m$. The arrow and closed circle indicate the specific DNA-protein complexes and free probes, respectively.

tion of A1 and A2, and the formation was competed by the addition of E1. The intensities of the two major bands were not affected by the addition of $A2^m$. The binding activity to E1 was detected in the dry seeds but was lower than for the other two motifs (data not shown). Therefore, it is likely that the same protein binds to the two probes and interacts with probe A1 more strongly.

The TGACGT motif-binding activity from the axes was also examined (Fig. 5). The activity for A1 was highest in the axes at 1 DAI. It decreased in the axes at 2 DAI, and disappeared by 4 DAI. In addition, there was only one band of complex between the DNA and protein, although two bands were detected in the nuclear proteins from the cotyledons. The activity was inhibited by the addition of A1 as a competitor. The addition of $A1^m$ had no effect on the formation of complex within A1. Thus, the nuclear protein binding to the TGACGT motif exists in axes until 2 DAI. The activity for A2 was highest in the axes at 1 DAI, but this activity was lower than for A1 and disappeared at 2 DAI. In contrast, α-amylase expression was detected in the axes at 4 DAI. Therefore, this protein would not be involved in the α-amylase expression in the axes.

Analysis of the promoter region by transient assay with particle bombardment

I examined whether the TGACGT motifs act as *cis*-elements conferring germination-specific gene expression. The promoter region of the α-amylase gene from *V. mungo* was fused to a coding region of the β-glucuronidase (GUS) gene from *Escherichia coli.* I also constructed deletion mutants of the promoter region fused to the GUS gene. These GUS fused genes were delivered into the cotyledons at 1 DAI by particle bombardment, because the α-amylase mRNA level increased by 2 DAI drastically as shown in Fig. 1, suggesting that transcription of the gene is most active in the cotyledons from 1 DAI to 2 DAI. pBI221, in which the CaMV35S promoter was fused to the GUS gene, was used as a positive control. pDO432 in which the CaMV35S promoter was fused to the firefly luciferase (LUC) gene, was used as an internal control. The promoter region from –639 to +29 gave 60% activity compared to that of pBI221 (Fig. 6). The deletion mutant of –453 showed a reduction in the GUS activity, but the activity was maintained on deletion up until –135 of the promoter region. However, deletion until –103 of the promoter region reduced the GUS activity markedly. The activity on deletion until –44 of the promoter region was the same as that until –103. Therefore, the sequence from –135 to –104 is required for high-level expression of the α-amylase gene in germinated cotyledons. The TGACGT motif located at –125 was mutated in the region from –135 to +29 and the mutation reduced the GUS activity to one-third that of pAmy-135. This result confirmed that the motif acts as a *cis*-acting element for the gene expression. I examined whether the TGACGT motif is important for the gene expression in the axes of the seedlings of *V. mungo*. The region from –639 to +29 fused to the GUS gene gave strong

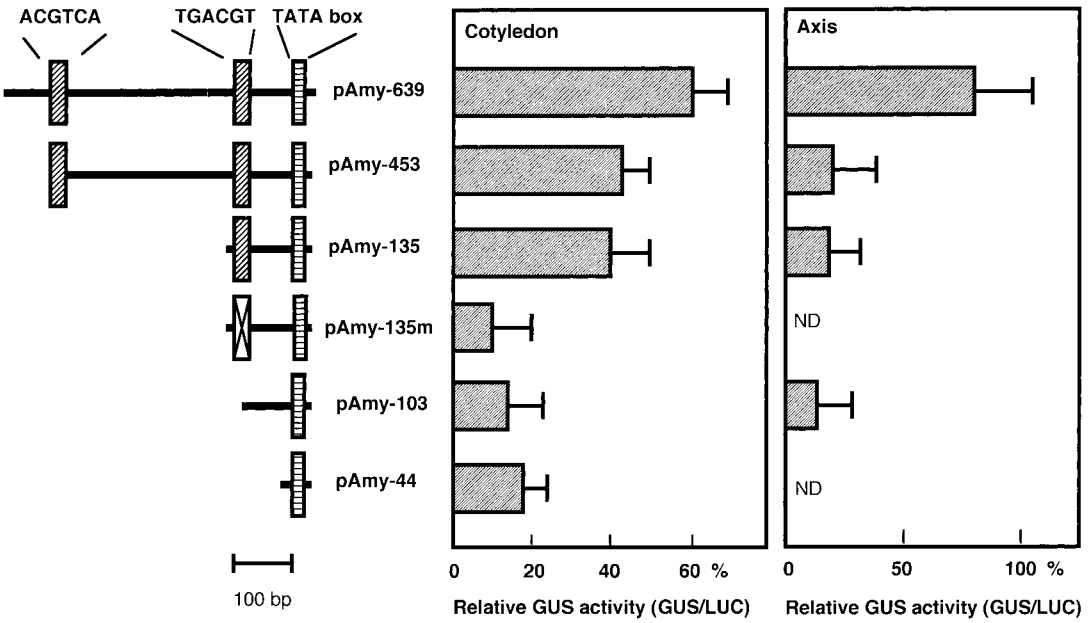

Fig. 6 Deletion analysis of the promoter region of the α-amylase gene. Deletion mutants of the 5′-promoter region were fused to the GUS reporter. pAmy-135m includes the TGACGT motif mutated by PCR. The chimeric GUS genes were delivered into the cotyledons (Cotyledon) or axes (Axis) of *V. mungo* seedlings by particle bombardment. pDO432, in which the CaMV35S promoter is fused to the LUC gene, was used as an internal control. The ratio of GUS to LUC was calculated by comparing the ratio of cotyledons or axes that were bombarded with pBI221. The results shown are means and standard errors from three or more independent bombardments. ND: not done.

activity in the axes, but deletion until –453 reduced the activity markedly. Therefore, the sequence from –639 to –454 is required for the gene expression in the axes. Regulatory elements for the expression in the promoter region of the α-amylase gene from *V. mungo* may differ between the cotyledons and axes in seedlings.

Discussion

There are two TGACGT motifs in the promoter of the α-amylase gene from *V. mungo*. Both interacted with the nuclear proteins in the cotyledons of germinated seeds, but the TGACGT motif at –125 was indicated to be a positive regulatory element in the germinated seeds. This motif is found in the promoter regions of several genes. The CaMV35S promoter is known to give high activity in any organ (Ow et al. 1987). A *cis*-element in the CaMV35S promoter, designated as-1, contains TGACGT and TGACGC. These sequences bound to the TGA transcription factors (Katagiri et al. 1989). The two motifs are required for strong promoter activity, and deletion of TGACGT reduced the promoter activity (Ow et al. 1987). In addition, the minimum promoter fused to four copies of as-1 showed a high level of transcriptional activity in the leaf in addition to the root (Lam and Chua 1990). These facts suggest that one copy of the TGACGT motif with the minimum promoter does not give strong promoter activity in germinated seeds. Therefore, the other sequence located downstream from –120 may be required for strong expression specific to seed germination.

Fig. 3 shows that two bands were detected in the dry seeds and the low mobility band disappeared at 2 DAI. The high mobility band was detected until 3 DAI, although its activity was low on 3 DAI. At least two types of proteins may have interacted with the TGACGT motifs and formed these two bands of protein–DNA complex, because generally TGA transcription factors are known to be a small gene family (Katagiri et al. 1989, Xiang et al. 1997). I need to isolate the cDNA encoding TGA factor from *V. mungo* to elucidate the mechanism of the expression of the α-amylase gene.

Activity of the nuclear protein was not detected in the immature seeds, and was highest in the dry seeds, decreasing during germination (Fig. 3). This pattern did not correspond to the expression of the α-amylase gene (Fig. 1). It is possible that transcription factors synthesized after imbibition of the seeds activate the α-amylase gene expression in cooperation with TGA factors from quiescent seeds. In addition, I speculate that negative regulators are involved in the gene expression in addition to positive regulators, because the transcription factor VP1 and the protein kinase, PKABA1, are known to repress the expression of the α-amylase gene in monocot seeds (Hoecker et al. 1995, Gómez-Cadenas et al. 1999). I also postulate that the negative regulators synthesized late in the seed maturation disappeared after imbibition of the seed, and that transcription of the gene is also activated.

A transient assay by particle bombardment indicated that deletion of the region from –639 to –454 reduced the promoter activity of the α-amylase gene in the axes of seedlings of *V. mungo* (Fig. 6). When the seedling of *V. mungo* is grown in the dark, starch stored in the cotyledon is consumed and carbon starvation occurs in the axis. α-Amylase expression in bean callus is known to result from carbon starvation (Kim et al. 1997). The expression in the callus was not affected by the addition of gibberellin, although gibberellin and an inhibitor of its biosynthesis have an effect on α-amylase expression in the germinating seeds of *V. mungo* (Taneyama et al. 1995). Thus, the mechanism behind the induction of α-amylase expression in the axes could be different from that in the cotyledons of germinated seeds and transcription factors also could differ between the two mechanisms.

Sugar represses the expression of the α-amylase gene in rice, and amylase element, TATCCA and G box element, ACGTG in the promoter region is involved in the sugar-dependent expression (Hwang et al. 1998, Lu et al. 1998, Toyofuku et al. 1998). Although the promoter region of the α-amylase gene of *V. mungo* does not contain the G box, three amylase element-related sequences, TATCTC at –333 and –258 and TATCCT at –217 are found in the promoter (Fig. 2). However, the present results suggest that the region from –639 to –454 is required for expression in the axes. If the three amylase element-related sequences confer the expression in the axes, some elements in the region from –639 to –454 regulate the activation of the expression synergistically. The repeat of TACGTA was found at –525 and –507 (Fig. 2), and these are candidates for the *cis*-elements in the region from –639 to –454. Culture cells are useful for analyzing sugar regulation of the expression of α-amylase, because *cis*-elements for sugar starvation of the rice α-amylase gene have been identified in rice suspension cells (Hwang et al. 1998, Lu et al. 1998) and callus (Toyofuku et al. 1998). Therefore, the GUS gene fused to the promoter of the *V. mungo* α-amylase gene should be introduced into the callus to establish the *cis*-elements for sugar starvation.

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