Title: The Isolation of cDNAs of Gibberellin Regulated Genes from Azuki Bean Epicotyls

Author: Tsuyoshi Kaneta

Department of Biology, Graduate School of Science, Osaka University

Contents

Abbreviations

GA_3	gibberellic acid
IAA	3-indoleacetic acid
DMSO	dimethylsulfoxide
FITC	fluorescein isothiocyanate
MT(s)	microtubule(s)
PBS	phosphate buffered saline
mRNA	messenger ribonucleic acid
cDNA	complementary deoxyribonucleic acid
Tris	Tris(hydroxymethyl)amino-methane
EDTA	ethylenediaminetetraacetic acid
ACC	1-aminocyclopropane-1-carboxylate
AOA	amino-oxyacetic acid
SAM	*S*-adenosylmethionine
2,4-D	2,4-dichlorophenoxyacetic acid
BA	6-benzylaminopurine
MES	2-(N-morpholino)ethanesulfonic acid, monohydrate

Summary

Gibberellins promote the longitudinal expansion and inhibit the lateral expansion of cells in stems of various higher plants. Gibberellins have been reported to promote stem elongation in the intact plants, but often failed to promote the elongation of excised stem segments. In azuki bean seedlings, however, GA_3 applied together with IAA promotes the elongation induced by IAA (Shibaoka 1972). Thus, I chose excised segments of azuki bean epicotyls as plant materials and examined the effect of actinomycin D, an inhibitor of transcription, on GA_3-promotion of IAA-induced elongation. GA_3 altered the orientation of cortical microtubules (MTs) transversely to the cell axis during the elongation caused by GA_3 together with IAA, and it was suggested that GA_3 regulates the direction of cell expansion by regulating the orientation of cortical MTs (Shibaoka 1974). I also investigated whether or not GA_3-induced gene expression was involved in GA_3-induced reorientation of cortical MTs.

When epicotyl segments taken from azuki bean seedlings were pretreated with GA_3 and then incubated with IAA, GA_3 promoted the elongation caused by IAA and brought about a predominance of transverse cortical MTs in the epidermal cells of the segments. The change in the arrangement of MTs caused by pretreatment with GA_3 was evident 1 h after the start of subsequent incubation with IAA when the effect of pretreatment with GA_3 on the elongation had not yet become apparent. Pretreatment with GA_3 did not cause any change in the arrangement of MTs when GA_3-pretreated segments were not incubated subsequently with IAA. Although actinomycin D applied before treatment with IAA did not inhibit the IAA-induced elongation, the drug diminished the promotion of the elongation caused by pretreatment with GA_3 and prevented GA_3 from bringing about a predominance of transverse MTs when the drug was applied during the pretreatment with GA_3 (Part I, Kaneta et al. 1993).

Since these results suggested that GA_3-induced synthesis of mRNA was involved in the promotion by GA_3 of IAA-induced elongation and in the GA_3-induced rearrangement of cortical MTs, differential screening was done with an intention to isolate cDNA clones of mRNAs the accumulations of which were altered by GA_3, which led me to the isolation of a cDNA clone of mRNA the

amount of which was decreased by GA_3. The predicted product encoded by this cDNA had close similarity to 1-aminocyclopropane-1-carboxylate (ACC)-oxidases identified in other plant species in amino acid sequence (about 80% identity). Thus the gene was designated *AB-ACO* (**a**zuki **b**ean **ACC**-**o**xidase). GA_3 also decreased the activity of ACC-oxidase in azuki bean epicotyls, but it did not decrease the ethylene evolution. GA_3 increased the ethylene evolution and ACC contents. GA_3 seems to increase the ethylene production through promoting the synthesis of ACC. The decreases in the accumulation of *AB-ACO* mRNA and in the activity of ACC-oxidase by GA_3 may partially compensate the decrease in the elongation of epicotyls caused by ethylene whose production is increased by GA_3 (Part II).

By differential screening, five cDNA clones of mRNAs the amounts of which were increased by GA_3 were also isolated. These genes were named *GAUR1,2,3,4,5* (**GA**$_3$ **u**p-**r**egulated gene). Northern blot analyses showed that the effects of GA_3 on the accumulations of *GAUR1,2,3,4* mRNA were evident after 0.5-h incubation. The effect of GA_3 on the accumulation of *GAUR5* mRNA was not visible after 2-h incubation and was evident after 6-h incubation. Sequence analyses of *GAUR* cDNA clones indicated that *GAUR4* and *GAUR5* were similar to *GAST1* and γ-TIP respectively, which had been reported as genes the expressions of which were promoted by GA_3. *GAUR1* was partly similar to *SCARECROW* gene, which was suggested to be a transcription factor, identified in *Arabidopsis* mutant. C-terminal region of *GAUR2* predicted product was similar to entire sequence of DNA-3-methyladenine glycosidase from bacteria. *GAUR3* had extensive similarity to proline-rich cell wall protein from cotton fiber and a potential transmembrane segment in C-terminal region which interact plasma membrane (Part III).

The use of transgenic plants is essential for the characterization of genes isolated, but the method for the preparation of transgenic azuki bean plants had not been established. Therefore, experiments were done to find out conditions which allow callus, induced from a small piece of azuki bean epicotyl, to regenerate shoots. Calli, which had generated on a medium that contained 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin and 6-benzylaminopurine (BA) as cytokinin, regenerated shoots on transplanting calli to a medium that

contained IAA as auxin, and *trans*-zeatin as cytokinin. The regenerated shoots grew into whole plants. The concentration of 2,4-D in callus-inducing medium could not be lower than 10 μg/l and higher than 10 μg/l. Studies to generate transgenic azuki bean plants are now in progress (Part IV).

General Introduction

Higher plants have the ability to regulate their shape by regulating the way of their development to adapt themselves to the environment. The control of the direction of cell expansion is the important feature of the environmental control of the plant morphogenesis. Plant hormones, such as auxin, gibberellins and ethylene, regulate the shape of cells in stems of higher plants. The shape of stem cells seems to be determined by the balance of these plant hormones. Therefore, the clarification of the mechanism of action of plant hormones at a molecular level is indispensable for full understanding of the regulation of plant morphogenesis. Gibberellins have been known to promote the longitudinal expansion and inhibit the lateral expansion of cells in stems of various higher plants. However, the mechanism of cell elongation induced by gibberellins has not yet been clarified at the molecular level.

Azuki bean seedlings possess merit as plant material for studies on the effects of gibberellins on stem elongation. Gibberellins promote the elongation of stems in various plant species when they are applied to intact plants, but they often fail to promote the elongation of excised stem segments. In azuki bean seedlings, however, GA_3 promotes the elongation of excised stem segments. Although GA_3 applied alone causes no elongation in azuki bean epicotyl segments, GA_3 applied together with IAA promotes the elongation induced by IAA. Therefore, I tried to examine the mechanism of GA_3-induced promotion of IAA-induced elongation at a molecular level in the segments of azuki bean epicotyls in the present study.

Part I

Involvement of GA_3-induced gene expression in GA_3-induced elongation of azuki bean epicotyls and the reorientation of cortical microtubules

Introduction

Direction of plant stem cell expansion is determined by the orientation of cellulose microfibrils in the cell wall (Green 1980), which, in turn, is considered to be controlled by cortical microtubules (MTs); (Giddings and Staehelin 1991).

Plant hormones are known to be involved in the control of the shape of plants via control of the direction of cell expansion. Moreover, all major plant hormones have been shown to have the ability to alter the arrangement of cortical MTs (Shibaoka 1994). Gibberellins, which promote elongation in the absence of lateral expansion of cells in stems of plants, have been reported to cause cortical MTs to orient themselves transversely to the cell axis (Shibaoka 1994, Ishida and Katsumi 1992, Sakiyama-Sogo and Shibaoka 1993), and it has been suggested that gibberellins regulate the direction of cell expansion by regulating the orientation of cortical MTs. In spite of repeated demonstrations of the ability of gibberellins to alter the orientation of cortical MTs, the mechanism by which gibberellins alters the orientation of MTs has not been clarified. Although numerous studies have been done to demonstrate that gibberellins induce the expression of genes in plant tissues that include the shoots of maize, pea (Chory et al. 1987) and dwarf tomato plants (Shi et al. 1992), little has yet been done to examine whether or not the control by gibberellins of cell expansion and of the orientation of cortical MTs involves the gibberellin-induced expression of specific genes.

The present study was undertaken to examine whether or not the gibberellin-induced expression of genes is required for the control of the direction of cell expansion and the orientation of cortical MTs by gibberellins. I examined the effects of actinomycin D, an inhibitor of transcription, on the GA_3-induced elongation and the reorientation of cortical MTs in epicotyls of azuki bean seedlings. I found that the GA_3-induced expression of genes appears to be involved in the control by GA_3 of both cell expansion and the rearrangement of MTs.

Materials and Methods

Plant material — Seedlings of the azuki bean (*Vigna angularis* 'Takara-wase') were grown under continuous light (4,000 lux) at 27 °C for 5 days. The seedlings were kept at 15°C overnight before use to increase the sensitivity of the seedlings to GA_3 (Nakamura and Shibaoka 1980).

Treatments — Epicotyl segments, 10 mm in length, were obtained by cutting azuki bean epicotyls at sites 5 mm and 15 mm below the node of the first leaves. Ten 10-mm epicotyl segments were floated on 4 ml of basal medium (10 mM potassium phosphate buffer, pH 6.8, containing 3 % sucrose), with or without substances to be tested, and incubated in the light (4,000 lux) at 27 °C for 18 h. Then the lengths of segments were measured.

For experiments in which epicotyl segments were pretreated with GA_3 and/or actinomycin D, ten 12-mm epicotyl segments (from 5 mm to 17 mm below the node of the first leaves) were floated on 4 ml of basal medium, with or without substances to be tested, and incubated in the light (4,000 lux) at 27 °C for 1 h. The segments were then washed by immersing them in basal medium for 7 min with occasional agitation and 10-mm segments were prepared by cutting small pieces from both ends of the 12-mm segments. The ten resultant 10-mm segments were then incubated in basal medium with or without 100 μM IAA. After appropriate time intervals, the lengths of these segments were measured again and the orientation of cortical MTs in the epidermal cells were examined. IAA and GA_3 were used at 100 μM in all experiments. Actinomycin D was dissolved in DMSO at 30 mM for preparation of a stock solution and it was used at 30 μM or lower concentrations. DMSO was added to each test medium at 0.1 % and to the control medium for each experiment. Pretreatment of segments with actinomycin D was started 10 min before the start of treatment with GA_3 to avoid the possibility that GA_3 might exert its effect before actinomycin D had penetrated the segments.

Immunofluorescence microscopy — The arrangement of cortical MTs in epidermal cells was examined by immunofluorescence

microscopy according to the methods of Sakiyama and Shibaoka (1990) and Hogetsu and Oshima (1986). Segments were cut in half and the upper half of each segment was fixed with 3.7 % formalin in 50 mM potassium phosphate buffer (pH 7.3) that contained 0.1 % Nonidet P-40 and 10 % DMSO at room temperature for 1 h. The fixed half-segments were frozen on a freezing stage at about -35 °C. Tangential longitudinal sections, about 50 μm in thickness, were cut from the basal regions of the frozen half-segments with a sliding microtome. MTs were stained by the method of Wick et al. (1981). In brief, the sections were washed with phosphate-buffered saline (PBS; pH 7.4) and incubated with mouse monoclonal antibody against chicken brain α-tubulin, which had been diluted 1:500 in PBS, at 37 °C for 1 h. Then the sections were washed with PBS and stained with FITC-conjugated antibodies raised in rabbit against mouse IgG, which had been diluted 1:10 in PBS, at 37 °C for 1 h. After washing with PBS, the stained sections were mounted with a mixture of PBS and glycerol (1:1, v/v) that contained 0.1 % *p*-phenylenediamine and examined with a microscope equipped with epifluorescence illumination. An EY455 was used for excitation filter and a G520 filter was used as a barrier filter. Photomicrographs of MTs in epidermal cells were taken on Kodak T-max 400 film.

Arrangement of MTs — The orientation of cortical MTs adjacent to an outer tangential wall of each epidermal cell was examined on the photomicrographs and histograms were constructed to show the percentages of cells with MTs within a specific range of angles to the cell axis, as follows: 0-30° (longitudinal, L); 30-60° (oblique, O); and 60-90° (transverse, T). The percentage of cells with MTs with mixed orientations is indicated as R (random) in the histograms. For each histogram, data from 252 to 417 cells from ten segments were collected. Data from stomatal guard cells and the surrounding cells, which had the cell-specific MT-arrangement, were not included in the construction of histograms (Sakiyama and Shibaoka 1990).

Results

Effects of actinomycin D on the GA_3-induced elongation of segments — As reported many years ago (Shibaoka 1972), GA_3 at 100 μM promoted the elongation of epicotyl segments that was induced by 100 μM IAA (Table 1). Actinomycin D at 30 μM, applied to the segments together with IAA and GA_3, greatly inhibited the elongation caused by these two hormones (Table 1). Actinomycin D, at this same concentration, also inhibited the elongation caused by IAA alone.

Pretreatment with 100 μM GA_3 promoted the elongation of segments that was induced by 100 μM IAA applied after pretreatment with GA_3 (Table 2). Promotion of the elongation by pretreatment with GA_3 was almost completely eliminated by actinomycin D applied at 30 μM during the pretreatment with GA_3 (Table 2). Actinomycin D at 1.0 μM was effective in reducing the effects of pretreatment with GA_3, but it was ineffective at 0.3 μM (data not shown). Actinomycin D at 30 μM, applied prior to treatment with IAA, did not inhibit the elongation induced by subsequent incubation with IAA (Table 2).

A set of segments was pretreated with basal medium with or without 100 μM GA_3 and then incubated in basal medium with or without 100 μM IAA. Other sets of segments were pretreated with basal medium plus 30 μM actinomycin D or plus 30 μM actinomycin D and 100 μM GA_3 before incubation with 100 μM IAA. The lengths of the segments were measured at appropriate intervals (Fig. 1). Pretreatment with GA_3 has no effect on the elongation of segments when pretreated segments were not subsequently incubated with IAA. However, as already shown in Table 2, GA_3 applied before treatment with IAA greatly promoted the elongation caused by IAA. The promotion of elongation by pretreatment with GA_3 was clearly apparent 2 h after the start of subsequent treatment with IAA (significant at the 0.1 % level), but not after only 1 h (Fig. 1). The absence of any difference in length between segments that had not been pretreated with GA_3 and GA_3-pretreated segments after a subsequent 1-h incubation with IAA was repeatedly confirmed in five separate experiments (data not shown). Inhibition by actinomycin D of promotion by GA_3 of IAA-induced elongation is illustrated in Figure 1. Unlike the case in the experiment for which data are shown in Table 2,

pretreatment with actinomycin D decreased the elongation caused by subsequent incubation with IAA by about 20 % in the experiment for which results are shown in Figure 1. The sensitivity of epicotyls to pretreatment with actinomycin D seemed to *differ* among batches of seedlings.

Effects of actinomycin D on the GA_3-induced reorientation of cortical MTs — The arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells differed from one cell to another in the segments before treatment (Fig. 2). In some cells the cortical MTs were oriented longitudinally and in others they were oriented obliquely or transversely. The percentages of cells with longitudinal MTs, with oblique MTs and with transverse MTs were almost the same (Fig. 3). Treatment with 100 μM GA_3, with 30 μM actinomycin D, with GA_3 plus actinomycin D, and with basal medium alone did not significantly alter the arrangement of cortical MTs. However, the percentage of cells with transverse MTs tended to decrease in all cases (Fig. 4). The pretreated segments were subsequently treated with 100 μM IAA or with basal medium alone and the arrangement of cortical MTs was examined after incubation for 0.5, 1.0 and 2.0 h with IAA (Figs. 5, 6 and 7). The treatment with IAA for 0.5 h did not cause any significant changes in the arrangement of MTs (Fig. 5). However, the percentage of cells with transverse MTs, which tended to decrease during the pretreatment, tended to increase in segments that had subsequently been incubated with IAA. GA_3 applied before IAA brought about a predominance of transverse MTs and this effect of GA_3 was evident 1.0 h after the start of treatment with IAA (Fig. 6d). The effect of pretreatment with GA_3 on the orientation of cortical MTs was greatly reduced by the simultaneous application of actinomycin D (Fig. 6f). The effects of pretreatment with GA_3 on the arrangement of cortical MTs and the effects of actinomycin D on the GA_3-induced rearrangement of cortical MTs were more clearly observed 2.0 h after the start of treatment with IAA (Fig. 8). The percentage of cells with transverse MTs was 76 % in segments that has been pretreated with GA_3 and then incubated with IAA (Fig. 7d), while the percentage was 33 % in segments that had not been pretreated with GA_3 but were incubated with IAA (Fig. 7c) and it was 36 % in segments that had been pretreated with actinomycin D together

with GA_3 and then incubated with IAA (Fig. 7f). Pretreatment with GA_3 alone did not bring about a predominance of transverse MTs (Fig. 8b). The percentage of cells with transverse MTs was 14 % in segments that had been pretreated with GA_3 and subsequently incubated in basal medium (Fig. 7b). The percentage of cells with longitudinal MTs increased during the incubation in basal medium (Fig. 6a, b and Fig. 7a, b), suggesting that the depletion of IAA causes a change in the orientation of MTs.

Discussion

Actinomycin D applied together with IAA and GA_3 inhibited the elongation of segments of azuki bean epicotyls that was otherwise caused by these two hormones. This result does not, however, imply that transcriptional processes are involved in the promotion by GA_3 of the elongation of epicotyls, because actinomycin D also inhibited the elongation caused by IAA alone. We can not exclude the possibility that actinomycin D inhibited the elongation of the segments by inhibiting IAA-induced processes or processes which were necessary for cell expansion itself. Thus, we can not draw any relevant conclusions from the results of experiments in which actinomycin D was added together with IAA and GA_3. Therefore, I designed experiments in which segments were treated with GA_3 and IAA sequentially and actinomycin D was applied only during the treatment with GA_3. As reported earlier (Shibaoka 1972), pretreatment with GA_3 promoted the elongation caused by subsequent incubation with IAA (Table 2). Actinomycin D applied during the pretreatment with GA_3 eliminated the GA_3-induced promotion of epicotyl elongation, whereas pretreatment with actinomycin D did not reduce the elongation caused by IAA (Table 2). Pretreatment with actinomycin D did, however, occasionally slightly inhibit the elongation caused by subsequent treatment with IAA (Fig. 1). For the time being, I cannot provide any convincing explanation for this inhibition. It is possible that actinomycin D inhibited the effects of endogenous gibberellins or that a small fraction of the actinomycin D applied during the pretreatment remained in the segments and this residual actinomycin D inhibited the effect of subsequently applied IAA.

Pretreatment with GA_3 brought about a predominance of transverse MTs in epidermal cells of epicotyl segments that were subsequently incubated with IAA (Fig. 6d, 7d and 8d), and the effect of the pretreatment with GA_3 was reversed by simultaneously applied actinomycin D (Fig. 6f, 7f and 8f), suggesting that the GA_3-induced expression of genes is involved in the GA_3-induced rearrangement of cortical MTs. Pretreatment with GA_3 alone did not bring about a predominance of transverse MTs (Fig. 4b). GA_3 alone, or GA_3-induced transcripts alone, do not appear to have the ability to alter the orientation of cortical MTs

and an auxin is required if they are to have an effect on the arrangement of MTs. Two possibilities can be suggested for the role of auxin. In one case, auxin itself is required and, in the other, the auxin-induced expansion of cells is required. Since no rearrangement of cortical MTs was evident before elongation started in segments of azuki bean epicotyls, the latter possibility cannot be ruled out. In this connection, it should be mentioned that auxin has been reported to change the orientation of cortical MTs before the start of the elongation in mesocotyls of maize (Nick et al. 1990) and in hypocotyls of radish (Sakoda et al. 1992).

GA_3 affects the arrangement not only of cortical MTs but also of newly laid down cellulose microfibrils, causing them to be oriented transversely to the cell axis (Takeda and Shibaoka 1981). Thus, it appears that cortical MTs regulate the orientation of cellulose microfibrils. According to Giddings and Staehelin (1988), cortical MTs should be associated with the plasma membrane if they are to regulate the orientation of newly laid down cellulose microfibrils and, therefore, cortical MTs are considered to be associated with the plasma membrane before and after their reorientation. Thus, the reorientation of cortical MTs by GA_3 seems to involve the dissociation from and the reassociation with the plasma membrane of cortical MTs. Since GA_3 has been reported to alter the stability of cortical MTs when it alters their orientation (Mita and Shibaoka 1984, Akashi and Shibaoka 1987, Sakiyama and Shibaoka 1990), it seems highly probable that the nature of the association of MTs with the plasma membrane changes during the GA_3-induced reorientation of MTs.

The present study indicates that GA_3-induced transcripts are involved in the GA_3-induced promotion of elongation of azuki bean epicotyls. The identification of the transcripts themselves and the products of translation of the transcripts are of crucial importance as I continue to characterize the mechanism by which GA_3 promotes stem elongation.

Part II

GA_3 down-regulated gene

Introduction

Gibberellins regulate the shape of cells in stems of higher plants. They promote the longitudinal expansion and inhibit the lateral expansion of cells in stems of various higher plants (Shibaoka 1993).

In recent years, candidates for molecules related to the promotion of elongation by gibberellins were isolated by differential screening. For example, *GAST1* gene was isolated from shoots of tomato (Shi et al. 1992), γ-TIP from shoots of *Arabidopsis* (Phillips and Huttly 1994), *CRG16* gene from hypocotyls of cucumber (Chono et al. 1996) and *sam* gene from corollas of petunia (Izhaki et al. 1996). However, there is no direct evidence which relates the isolated genes to the promotion of stem elongation by gibberellins, that is, the mechanism of cell elongation induced by gibberellins has not yet been clarified at the molecular level.

In azuki bean epicotyl segments, GA_3 applied before treatment with IAA promotes IAA-induced elongation. Since actinomycin D, an inhibitor of transcription, greatly suppresses the effect of GA_3 applied before treatment with IAA (Part I), GA_3-induced synthesis of mRNA seems to be involved in GA_3-induced promotion of IAA-induced epicotyl elongation. Therefore, I tried to isolate genes the expressions of which were changed by GA_3. In this Part, I describe the isolation of cDNA, the amount of mRNA of which was decreased by GA_3. The sequence analysis of the cDNA indicated that the gene was a gene for ACC-oxidase, which catalyze the final step of ethylene biosynthesis, in azuki bean epicotyls. I also examined the effect of GA_3 on ethylene synthesis in the segments of azuki bean epicotyls.

Materials and Methods

Plant material — Seeds of azuki bean were sown in moistened vermiculite. Seedlings were grown under continuous light (4,000 lux) at 27 °C for 5 days.

Treatments — Segments were made by cutting azuki bean epicotyls at sites 5 mm and 15 mm below the node of the first leaves. Twenty-five 10-mm epicotyl segments were floated on 2.5 ml of basal medium (10 mM potassium phosphate buffer, pH 6.2, containing 3% sucrose), with or without plant hormones in 30-ml Erlenmeyer flasks. In all experiments in this Part, flasks with the segments were sealed with silicone rubber stoppers and kept under continuous light at 25 °C. For each measurement of the lengths of the segments in the time course studies, each lot of 25 segments sealed in a flask was used and the segments once used for the measurement were no longer used for the later measurements.

Constructions of cDNA libraries from the azuki bean epicotyls — Total RNAs were extracted from epicotyl segments, which had been treated with basal medium that contained IAA or basal medium that contained IAA and GA_3 for 2 h, by SDS-phenol method by using Extract-A-Plant kit (CLONTECH Laboratories Inc.). Poly(A)$^+$ RNAs were enriched by using Oligotex-dT30 <super> (Nippon Roche Co., Ltd., Japan Synthetic Rubber Co., Ltd.) and cDNA libraries were constructed into λZAPII vector by using ZAP-cDNA SYNTHESIS KIT (STRATAGENE). 5×10^6 pfu. library was obtained from IAA-treated segments and 3×10^6 pfu. library from IAA plus GA_3-treated segments.

Differential screening — Each of the [α-^{32}P]dCTP-labeled cDNA probes prepared from poly(A)$^+$ RNA extracted from the segments treated with IAA for 2 h and from poly(A)$^+$ RNA extracted from segments treated with IAA plus GA_3 for 2 h was hybridized with each of the duplicated copies of the cDNA libraries on nylon membranes (Hybond-N, Amersham). The hybridization was performed in 5xSSPE (1xSSPE=10 mM NaH_2PO_4 pH 7.4, 150 mM NaCl, 1 mM EDTA) that contained 0.5 % SDS, 50 % formamide and 500 mg/ml salmon sperm DNA at 46 °C for 16 h. Then, the membranes were

washed once with 1xSSC (=150 mM NaCl, 15 mM *tri*-sodium citrate) that contained 0.1 % SDS at 68 °C, and three times with 0.2xSSC that contained 0.1 % SDS at 68 °C, and then the signals were detected with BAS-2000 system (FUJIFILM). The cDNA clones to be isolated were selected from $5x10^5$ plaques of cDNA libraries by comparing signals on each membrane. GA_3-dependence of expression of each of the isolated clones was reconfirmed by northern blot analysis. In this way, I obtained cDNA clones of mRNAs the accumulations of which were decreased or increased by treatment with GA_3. I focused my attention on one of these clones, which I named *AB-ACO*.

Screening of AB-ACO gene — *AB-ACO* clone isolated by differential screening was subcloned into pBluescript SK(-) vector according to *IN VIVO* Excision method by using ZAP-cDNA SYNTHESIS KIT (STRATAGENE). The insert excised with EcoRI and XhoI was labeled with [α-^{32}P]dCTP by Megaprime Labeling System (Amersham) and used to screen a library constructed from RNA extracted from segments treated with IAA. Forty cross-hybridizing plaques were excised as pBluescript SK(-) plasmids, and nine of them as well as the clone which was isolated by differential screening were used for sequence analyses.

Northern blot analysis — Ten μg of total RNA extracted from the segments treated with basal medium, basal medium with GA_3, basal medium with IAA, or basal medium with IAA plus GA_3 was separated on 1 % agarose gel, that contained 0.66 M formaldehyde, by electrophoresis. Gels were stained with ethidium bromide and the stained gels were photographed. Then, the separated samples were blotted on to nylon membranes (Hybond-N$^+$, Amersham). The synthesis of the probe, hybridization and washing of membranes were performed under the same conditions as described in '*Screening of AB-ACO gene*' section. Signals were detected with BAS-2000 system.

Sequence analysis — Sequencing of cDNA subcloned into pBluescript SK(-) vector was performed with the Dye Primer Cycle Sequencing Kit, FS (Perkin-Elmer Corporation) and ABI 373S DNA Sequencer (Applied Biosystems, Inc.).

ACC-oxidase activity — The activity of ACC-oxidase in epicotyl segments was measured as described by Hoffman and Yang (1982) and Hamilton et al. (1990). Before and immediately after treatment with basal medium or basal medium that contained plant hormones for 6 or 8 h, segments were immersed in 100 mM sodium phosphate buffer (pH 6.5) that contained 10 mM ACC and 10 mM amino-oxyacetic acid (AOA) and kept under vacuum for 2 minutes to infiltrate ACC and AOA into the segments. One hour later, segments were sealed in 5-ml glass vials and kept at 25 °C. One ml of gas in the vial was sampled after a further hour and the amount of ethylene in the sampled gas was measured by gas chromatography.

Measurement of ethylene evolution — One ml of gas was sampled from an Erlenmeyer flask, in which epicotyl segments had been floated on the basal medium or basal medium that contained plant hormones, every 2 h and the amount of ethylene in the sampled gas was measured by gas chromatography.

Measurement of ACC accumulation — ACC accumulated in epicotyl segments was extracted as described Philosoph-Hadas et al. (1996) and Jiao et al. (1986). Twenty-five epicotyl segments were homogenized with a glass homogenizer with 4 ml of 90 % ethanol at 70 °C. The homogenate was centrifuged at 15,000 g for 10 minutes and the resultant supernatant was saved and ethanol in the supernatant was evaporated under vacuum. The amount of ACC in the evaporation residue was measured as described by Lizada and Yang (1979). The residue was resolved in 600 µl of water, and then 300 µl of 10 mM $HgCl_2$ and 100 µl of a mixture of 5 % NaOCl and 10 N NaOH (2:1, v/v) were added to the aqueous solution of the residue. The resultant mixture was sealed in a 5-ml glass vial and kept on ice for 30 minutes. The amount of ethylene, which was produced from ACC, was measured by gas chromatography.

Results

Expression of the GA_3 down-regulated gene — I carried out a differential screening and isolated several cDNA clones the accumulations of mRNAs corresponding to which were changed by GA_3. In this part, I focused my attention on one of these clones the accumulation of mRNA corresponding to which was decreased by GA_3. Fig. 9 shows the effects of GA_3, IAA, and GA_3+IAA on time course of changes in amount of mRNA recognized by the ^{32}P-labeled probe prepared from this cDNA, as revealed by Northern blot analysis. Although, the mRNA of about 1.2 kb was barely detectable in epicotyls of intact plants (Fig. 9a, initial), it became apparent in excised epicotyl segments floated on basal medium within 2 h and its amounts continued to increase during 10-h incubation period (Fig. 9a, BM), suggesting that the accumulation of mRNA of this gene increased in response to the stimulus of wounding. IAA showed no effect on the expression of this gene: the time course change in the amount of the mRNA in segments treated with IAA was almost the same with that in the segments treated with basal medium (Fig. 9a, BM and +IAA). GA_3 decreased the wound-induced increase in the accumulation of mRNA of this gene, although GA_3 did not completely suppressed the increase (Fig. 9a, Initial, +GA_3). Either in the presence or in the absence of IAA, the accumulation of mRNA of this gene was smaller in GA_3-treated segments than in GA_3-untreated segments (Fig. 9a, compare BM with +GA_3, and +IAA with +IAA+GA_3).

Sequence of the AB-ACO cDNA — I isolated forty positive cDNA clones from 50,000 plaques of the unamplified cDNA library constructed from mRNAs from segments treated with IAA alone using the probe made from the cDNA of the GA_3 down-regulated gene that had been isolated by differential screening. The nucleotide sequences of nine of thus obtained clones and the original cDNA clone were determined. The longest three clones had identical sequence of 1184 bp (Fig. 10). This is in agreement with the result of Northern blot analysis in which about 1.2 kb mRNA was recognized.

The longest cDNA included the largest open reading frame (ORF, from 45-47 bp of ATG to 975-977 bp of TAG) which encoded 310 amino acids (Fig. 10). All ten cDNA clones, which I

sequenced, had sequence identical to that shown in Fig. 10, though their length was different. I could not find the iso-form of the cDNA.

The gene has extensive sequence similarity to 1-aminocyclopropane-1-carboxylate oxidases (ACC-oxidases) which had been identified in various higher plant species (about 80 % identity, more than 90 % similarity). Thus I designated this gene *AB-ACO* (**a**zuki **b**ean **ACC**-**o**xidase). Fig. 11 shows the amino acid alignments deduced from nucleotide sequences of genes of ACC-oxidase family, such as Pch313 from peach fruits (Callahan et al. 1992) which is most similar to AB-ACO (81 % identity, 92 % similarity), pGEFE (83 % identity, 94 % similarity) from geranium leaves (Wang et al. 1994), pTOM5 (82 % identity, 92 % similarity), pTOM13 (79 % identity, 92 % similarity) from tomato fruits (Holdsworth et al. 1987) and *AB-ACO* isolated from azuki bean epicotyls in this study.

Effect of GA_3 on ACC-oxidase activity — Since the product of *AB-ACO* gene, the expression of which was suppressed by GA_3, was presumed to be ACC-oxidase in azuki bean plants, I examined whether or not GA_3 caused the decrease in the activity of ACC-oxidase in segments of azuki bean epicotyls. Experiments were done both in the presence and in the absence of IAA (Table 3). As Table 3 shows, the activity of ACC-oxidase increased during the incubation with basal medium. It was doubled during the 6-h incubation and tripled during the 8-h incubation. IAA showed no effect on the wound-induced increase in the activity of ACC-oxidase. The difference in the activity of ACC-oxidase between segments treated with basal medium and those treated with basal medium that contained IAA observed after 6-h incubation was not significant at 10 % level (Table 3). On the other hand, GA_3 suppressed the increase in the activity of ACC-oxidase. Although the difference in the activity of ACC-oxidase between segments treated with basal medium and those treated with basal medium that contained GA_3 observed after 6-h incubation was not significant at 10 % level, that observed after 8-h incubation was significant at the 0.8 % level (Table 3). IAA alone had almost no effect on the activity of ACC-oxidase, but it greatly enhanced the effect of GA_3. IAA+GA_3 inhibited the increase in the activity by about 80 %, whereas GA_3 alone by about 30 % to 40 % (Table 3).

Effects of GA_3 on ethylene evolution and ACC accumulation — To explore the possibility that GA_3-induced suppression of the increase in the activity of ACC-oxidase brings about the decrease in ethylene evolution, I examined the effect of GA_3 on the ethylene evolution in azuki bean epicotyl segments in the presence and in the absence of IAA (Fig. 12). The rate of ethylene evolution in freshly prepared segments was low and the rate did not increase in segments treated with basal medium or basal medium that contained GA_3 alone (Fig. 12). On the other hand, the rate clearly increased in segments treated with basal medium that contained IAA (Fig. 12). The increase became evident during the first 2-h treatment and reached the maximum value during the fourth 2-h treatment, i. e. 6-8h (Fig. 12). GA_3 applied together with IAA enhanced the IAA-induced increase in the rate of ethylene evolution and the enhancement was remarkable during the fourth and the fifth 2 h, i. e. 6-8 h and 8-10 h (Fig. 12), in spite of the result that GA_3 and IAA synergistically inhibited the wound-induced increase in the activity of ACC-oxidase (Table 3). To provide an answer to the question of why GA_3 applied together with IAA increased the rate of ethylene evolution, whereas it caused the decrease in the activity of ACC-oxidase, I measured the content of ACC in epicotyl segments. The content of ACC in freshly prepared segments was low, and treatment with basal medium or basal medium that contained GA_3 scarcely caused the accumulation of ACC (Fig. 13). IAA alone increased the content of ACC in the epicotyl segments as it did in the rate of ethylene evolution and the accumulation reached the maximum value after 8-h treatment (Fig. 13). GA_3 applied together with IAA enhanced the accumulation of ACC caused by IAA (Fig. 13). The enhancement became distinct during the period between 4 h and 6 h (Fig. 13), when the enhancement by GA_3 of ethylene production was still not distinct (Fig. 12).

Effects of GA_3 and IAA on the elongation of azuki bean epicotyl segments — I examined the effect of GA_3 and/or IAA on the elongation of epicotyl segments under the conditions where I measured the rate of ethylene evolution (Fig. 14). Results were almost identical with those reported in Part I (Fig. 1). The GA_3-promotion of the elongation caused by IAA was evident 4 h after the start of treatment but not at 2 h (Fig. 14)

Discussion

An ACC-oxidase gene, pTOM13, was isolated at first as one of the ripening related genes from tomato fruits (Holdsworth et al. 1987). That pTOM13 gene coded ACC-oxidase was demonstrated by the results that antisense suppression of pTOM13 in transgenic tomato plants resulted in the decreases in the ethylene evolution and in the ACC-oxidase activity in leaf disks excised from the plants (Hamilton et al. 1990). Furthermore, function of this gene was directly confirmed in the way in which pTOM13 product expressed in yeast and oocytes of *Xenopus* was proved to synthesize ethylene from ACC (Spanu et al. 1991, Hamilton et al. 1991). Since *AB-ACO* gene has an extensive sequence similarity to pTOM13 gene and ACC-oxidase genes from other plant species (Fig. 11), I deduced that *AB-ACO* gene codes ACC-oxidase in azuki bean epicotyls.

I showed that GA_3 suppressed the increases in the accumulation of *AB-ACO* mRNA and in the activity of ACC-oxidase in the segments of azuki bean epicotyls (Fig. 9 and Table. 3). Since the suppression of the activity of ACC-oxidase was most distinct in segments treated with GA_3 together with IAA which showed most conspicuous elongation (Table. 3), I first thought that both the decrease in the ACC-oxidase activity and the promotion of elongation were regulated by the same GA_3-related system, in other words, the elongation by GA_3 was caused, at least partially, by the suppression of ACC-oxidase activity, because it had been known that ethylene acted oppositely to gibberellins: ethylene promoted the lateral cell expansion and inhibited the longitudinal cell expansion in stems, whereas gibberellins inhibited the former and promoted the latter. But, the results of experiments in which I examined the effects of GA_3 on ethylene evolution and on the accumulation of ACC noticed me that I was wrong. GA_3 applied together with IAA increased the rate of ethylene evolution (Fig. 12) and the accumulation of ACC in the epicotyl segments (Fig. 13). The decreases in the accumulation of *AB-ACO* mRNA and in the activity of ACC-oxidase do not seem to be responsible for the GA_3 promotion of epicotyl elongation.

I found that GA_3 increased the accumulation of ACC in the segments of azuki bean epicotyls (Fig. 13). In recent years, it has

been reported that GA_3 regulates the synthesis of *S*-adenosylmethionine (SAM), a precursor of ACC in the ethylene biosynthesis pathway. GA_3 increases the accumulation of SAM and the activity of SAM synthetase in dwarf pea epicotyls (Mathur and Sachar 1991, Mathur et al. 1993), and the accumulation of *sam* mRNA, which encodes SAM synthetase, is increased by GA_3 in petunia corollas (Izhaki et al. 1996). Thus, it is probable that the increase in the accumulation of ACC by GA_3, which I found in the segments of azuki bean epicotyls, was caused by the increase in the accumulation of SAM by GA_3. SAM is not merely a precursor of ACC. It also acts as a methyl-group donor in various transmethylation reactions and as a propylamine group donor in the polyamine biosynthesis pathway. Thus there is a possibility that the gibberellin-induced accumulation of SAM facilitates the gibberellin-induced stem elongation. Izhaki et al's result (1996) that *sam* gene is expressed at high level in elongating petunia stems and Boerjan et al's result (1994) that transgenic tobacco plants with reduced SAM synthetase activity show stunted phenotype may support this possibility. However, Boerjan et al. (1994) also reported that highly elevated SAM synthetase activity also led to the inhibition of stem elongation. SAM seems to be necessary for stem elongation but excess SAM seems to cause deleterious effect on stem elongation. If excess SAM causes the inhibition of stem elongation by increasing the amount of ACC and, therefore, ethylene evolution, gibberellin-induced decrease in the expression of ACC-oxidase may partially compensate the inhibition of stem elongation which is caused by gibberellin-induced increase in the amount of SAM. Although gibberellin-induced decreases in the accumulation of *AB-ACO* mRNA and in the activity of ACC-oxidase do not seem to be responsible for gibberellin-induced stem elongation, they undoubtedly contribute to gibberellin-induced stem elongation.

Besides *AB-ACO* cDNA clone, I obtained several cDNA clones of gibberellin up-regulated genes and down-regulated genes. Characterizations of such genes will be described in the next Part.

Part III

GA_3 up-regulated genes

Introduction

The promoting effects of gibberellins on stem elongation have been reported in various plant species. However, studies on molecular mechanisms of gibberellin-induced stem elongation have scarcely been done. I started the present study with an intention to identify genes responsible for gibberellin-induced stem elongation. In contrast to defect in studies on molecular mechanisms of gibberellin-induced stem elongation, numerous studies have been done on molecular mechanisms of gibberellin-induced enzyme synthesis in germinating seeds (Huttly and Phillips 1995). This is, at least partly, because gibberellins, which promote stem elongation in intact plants, often failed to promote the elongation of excised stem segments, while they induce enzyme synthesis in a tissue composed of single type of cells, aleurone cells. The use of intact plants that have organs other than elongating stems is not appropriate for the identification of genes responsible for gibberellin-induced stem elongation. I chose excised segments of azuki bean epicotyls as plant materials, because it has been reported that GA_3, which usually showed little effect on elongation of excised stem segments, markedly promoted elongation of azuki bean epicotyl segments (Shibaoka 1972). Thus, I performed differential screening by using excised azuki bean epicotyl segments and isolated GA_3 up-regulated genes.

Materials and Methods

Plant material — Azuki bean seedlings were grown under the same conditions as described in Part I *'Plant material'* section.

Treatments — Preparation of azuki bean epicotyl segments, 10 mm in length, and treatments with plant hormones were performed under the same conditions as described in Part I *'Treatments'* section.

Differential screening — Refer to *'Differential screening'* section in Part II.

Screening of GAUR genes — Probes prepared by using five kinds of *GAUR* cDNA were used to screen cDNA libraries as described in Part II *'Screening of AB-ACO gene'* section. For analyzing sequence of each of the *GAUR* genes, each three plaques that cross-hybridized with each of the five probes were used.

Northern blot analysis — Northern blot analysis were performed under the same condition as described in Part II *'Northern blot analysis'* section except that 5-μg, instead of 10 μg, total RNA was applied to agarose gel.

Sequence analysis — Refer to *'Sequence analysis'* section in Part II.

Results

Expression of the GA_3 up-regulated genes — I performed differential screening and isolated not only *AB-ACO* cDNA clone (Part II) but also five cDNA clones the accumulations of mRNAs corresponding to which were increased by GA_3. I named the genes corresponding to these five cDNAs *GAUR1,2,3,4,5* (**GA_3 up-r**egulated gene). Figures 15-19 shows the effects of GA_3, IAA, and GA_3+IAA on time course of changes in amounts of mRNAs recognized by the ^{32}P-labeled probes prepared from *GAUR1,2,3,4,5* cDNAs.

mRNA, recognized by the probe prepared from *GAUR1* cDNA was abundant in epicotyls of intact plants (Fig. 15, Initial). The amount of *GAUR1* mRNA in segments treated with basal medium decreased during the first 0.5-h incubation (Fig. 15, BM). Although, Fig. 15 shows that the decrease occurred during first 0.5-h incubation slightly recovered during following 1.5-h incubation, this recovery was unusual. In many cases, the amount of *GAUR1* mRNA was kept at low level in the segments treated with basal medium (data not shown). The amount of *GAUR1* mRNA in segments treated with basal medium that contained GA_3 was kept at the same level as that in intact plant (Fig. 15, +GA_3). The difference in the amount of *GAUR1* mRNA between segments treated with basal medium and those treated with basal medium that contained GA_3 observed after 0.5-h incubation was obvious and the difference was evident during 12-h incubation (Fig. 15, BM, +GA_3). IAA alone slightly diminished the decrease in the accumulation of *GAUR1* mRNA (Fig. 15, BM, +IAA). Treatment with basal medium that contained GA_3 plus IAA kept the amount of *GAUR1* mRNA and the amount of the mRNA in segments treated with basal medium that contained GA_3 plus IAA was slightly larger than the amount of mRNA in the segments treated with basal medium that contained GA_3 alone (Fig. 15, +IAA+GA_3).

GAUR2 mRNA was abundant in epicotyls of intact plants (Fig. 16, Initial). The accumulation of *GAUR2* mRNA greatly decreased during the first 0.5-h incubation, irrespective of the presence of plant hormone, to the level scarcely detectable by the method employed. It slightly increased during the following 5.5-h incubation in basal medium (Fig. 16, BM). The accumulation of the mRNA in segments treated with basal medium that contained GA_3 also greatly decreased during 0.5-h incubation, but was slightly

larger than that in segments treated with basal medium after 0.5-h incubation. The accumulation of *GAUR2* mRNA in segments treated with basal medium that contained GA_3 greatly increased during 12-h incubation (Fig. 16, $+GA_3$). IAA slightly inhibited the accumulation of *GAUR2* mRNA (Fig. 16, BM, +IAA). Treatment with GA_3 plus IAA increased the amount of *GAUR2* mRNA but the increase in the accumulation of the mRNA in segments treated with basal medium that contained GA_3 plus IAA was less than that in segments treated with basal medium that contained GA_3 alone (Fig. 16, $+GA_3$, $+IAA+GA_3$).

GAUR3 mRNA in intact plants was abundant (Fig. 17, Initial). The accumulation of *GAUR3* mRNA in segments treated with basal medium decreased during the incubation with basal medium (Fig. 17, BM). GA_3 kept the content of *GAUR3* mRNA at the initial level (Fig. 17, $+GA_3$). Although the difference in the amount of *GAUR3* mRNA between segments treated with basal medium and those treated with basal medium that contained GA_3 was small after 0.5-h incubation, it became large after 12-h incubation (Fig. 17, BM, $+GA_3$). IAA also kept the amount of *GAUR3* mRNA. However, the amount of *GAUR3* mRNA in segments treated with basal medium that contained IAA was smaller than that in segments treated with basal medium that contained GA_3 (Fig. 17, +IAA). The amount of *GAUR3* mRNA in segments treated with basal medium that contained IAA plus GA_3 was larger than that in segments treated with GA_3 (Fig. 17, $+IAA+GA_3$).

GAUR4 mRNA was abundant in epicotyls of intact plants (Fig. 18, Initial). During the first 0.5-h incubation, the accumulation of *GAUR4* mRNA decreased in segments treated with basal medium. The accumulation of *GAUR4* mRNA in segments treated with basal medium slightly increased during the following 1.5-h incubation (Fig. 18, BM). After 0.5 h, the amount of *GAUR4* mRNA in segments treated with basal medium that contained GA_3 was larger than that in segments treated with basal medium (Fig. 18, BM, $+GA_3$). The amount of *GAUR4* mRNA increased in segments treated with basal medium that contained GA_3 during 12-h incubation (Fig. 18, $+GA_3$). IAA had almost no effect on the accumulation of *GAUR4* mRNA (Fig. 18, +IAA). Treatment with basal medium that contained GA_3 plus IAA increased the accumulation of *GAUR4* mRNA, but the increase in the accumulation of *GAUR4* mRNA was smaller in segments treated

with basal medium that contained GA_3 plus IAA than in segments treated with basal medium that contained GA_3 alone (Fig. 18, $+GA_3$, $+IAA+GA_3$).

The amount of *GAUR5* mRNA was large in epicotyls of intact plants (Fig. 19, Initial). The amount of *GAUR5* mRNA in segments treated with basal medium was kept at the initial level during the first 2-h incubation, but it decreased thereafter (Fig. 19, BM). The amount of *GAUR5* mRNA in segments treated with basal medium that contained GA_3 was kept at the initial level during the first 6-h incubation and then decreased (Fig. 19, $+GA_3$). IAA had almost no effect on expression of *GAUR5* gene (Fig. 19, +IAA). The amount of *GAUR5* mRNA in segments treated with basal medium that contained IAA plus GA_3 was the same as that in segments treated with basal medium that contained GA_3 (Fig. 19, $+IAA+GA_3$).

Sequence of five GAUR cDNAs — For every *GAUR* gene, three cDNA clones were isolated from 100,000 plaques in the cDNA libraries constructed from mRNAs isolated from segments treated with IAA or those constructed from mRNAs isolated from segments treated with IAA plus GA_3 by using the probe prepared from each *GAUR* cDNA that had been isolated by differential screening. The nucleotide sequences of thus obtained clones and of the original cDNA clones were determined. The sequence of the longest cDNA clone for each *GAUR* gene was demonstrated.

The *GAUR1* cDNA clone included the largest ORF, from 324-326 bp of ATG to 1986-1988 bp of TAG, which encoded 554 amino acids (Fig. 20). The sequence of *GAUR1* has close similarities to *SCARECROW* gene (Laurenzio et al. 1996), which had been identified in *Arabidopsis* endodermis deficient mutant, in two regions (Fig. 25). Residues 245-304 of the *GAUR1* sequence, one of these two similar regions, showed similarity to residues 378-437 of the *SCARECROW* sequence which contained the region designated the VHIID domain (residues 394-435 of the *SCARESCROW* sequence; Laurenzio et al. 1996; Fig. 25, a), which is consensus region of several plant genes with unknown function. The *GAUR1* sequence had another region, residues 405-426, which was much similar to residues 530-551 of *SCARECROW* sequence (Fig. 25, b). Except for these two regions, the *GAUR1* sequence had no close similarity to the *SCARECROW* sequence: The

SCARECROW sequence has bZIP, transcription factors-like, domain but *GAUR1* sequence did not.

The *GAUR2* cDNA included the largest ORF, from 6-8 bp of ATG to 1176-1178 bp of TAG, which encoded 390 amino acids (Fig. 21). Residues from 210 to C-terminus of the *GAUR2* sequence had similarity to entire sequence of DNA-3-methyladenine glycosidase I gene which had been identified in *Escherichia coli* (Sakumi et al. 1986), *Haemophilus influenzae* (Fleischmann et al. 1995) and *Mycobacterium leprae* (Fig. 26). I could not find any genes which had the sequence similar to residues 1-210 of the *GAUR2* sequence.

The *GAUR3* sequence included the largest ORF, from 91-93 bp of ATG to 748-780 bp of TAG, which encoded 229 amino acids (Fig. 22). The *GAUR3* sequence has extensive similarity to cell wall proteins. For example, proline-rich cell wall proteins (John and Keller 1995) from cotton fiber and probable arabinogalactan protein precursor (Pogson and Davies 1995) from tomato fruits (Fig. 27). However, *GAUR3* had a potential transmembrane segment in C-terminal domain (Fig. 27, underline).

The *GAUR4* sequence included the largest ORF, from 37-39 bp of ATG to 364-366 bp of TGA, which encoded 109 amino acids (Fig. 23). The *GAUR4* sequence has extensive similarity to entire sequence of *GAST1* (Shi et al. 1992) from tomato shoot (Fig. 28), which was isolated as a gene whose expression was regulated by gibberellin. It is indicated that *GAUR4* gene was one of homologs to *GAST1* gene.

The *GAUR5* cDNA included the largest ORF, from 44-46 bp of ATG to 790-792 bp of TGA, which encoded 248 amino acids (Fig. 24). The *GAUR5* sequence has extensive similarity to entire sequence of γ-TIP, which functions as water channel, isolated as a gibberellin-regulated gene by differential screening in *Arabidopsis* shoot (Phillips and Huttly 1994).

Discussion

I isolated five cDNAs of mRNAs the accumulations of which increased by GA_3 in azuki bean epicotyls. Although I have not yet obtained any evidences for involvement of these genes in GA_3-induced stem elongation, I will try to discuss some possibilities of the participation of these genes in GA_3-induced stem elongation based on the results of sequence analysis and on time course changes in the accumulations of mRNAs.

The promoting effect of GA_3 on IAA-induced elongation was not evident after 1-h incubation (Part I, Fig. 1). If the expressions of *GAUR* genes are involved in GA_3-induced promotion of IAA-induced elongation, the accumulations of *GAUR* mRNAs should be induced by GA_3 earlier than the GA_3-induced promotion of IAA-induced elongation become evident. The effect of GA_3 on the accumulation of *GAUR1* mRNA was observed as early as after 0.5 h (Fig. 15). The amount of *GAUR2* mRNA decreased in not only GA_3-untreated segments but also in GA_3-treated segments during the first 0.5-h incubation, but the amount remained was slightly larger in GA_3-treated segments than in GA_3-untreated segments (Fig. 16). The difference in the amount of *GAUR3* mRNA between GA_3-untreated segments and GA_3-treated segments was small after 0.5-h incubation but visible (Fig. 17). After 0.5 h, the amount of *GAUR4* mRNA in GA_3-treated segments was larger than that in GA_3-untreated segments (Fig. 18). Although these results showed that the effects of GA_3 on the accumulations of *GAUR1,2,3,4* mRNA were evident before GA_3-promotion of IAA induced elongation became evident, there is no evidence for the involvement of *GAUR* genes in GA_3-promotion of IAA induced elongation. To examine the functions of these genes as candidates for molecules which relate to GA_3-promotion of IAA induced elongation, the use of transgenic plants is essential. I am trying the preparation of transgenic azuki bean plants with a view to characterize *GAUR* genes (next Part).

GAUR5 can not induce GA_3 induced promotion of IAA induced stem elongation, because GA_3-caused increase in the accumulation of *GAUR5* mRNA became evident after GA_3-promotion of IAA-induced elongation became evident (Fig. 19). *GAUR5* had sequence similarity to γ-TIP, which was presumed to be a water channel (Phillips and Huttly 1994, Maurel et al. 1993). The direction of

stem expansion was determined by cell wall of epidermal cells, while enlargement of stem is caused by water uptake of cells in cortex. I think that *GAUR5* may contribute to stem elongation by facilitating water uptake of cells in cortex.

I think, I should examine the localizations of the transcripts and the early time changes of the expression in five *GAUR* genes.

Part IV

Shoot induction from callus

Introduction

Plant cells have so called totipotency and, therefore, every cell can develop into an individual plant. Usually, single plant cells develop into individual plants via callus formation. That single plant cells, as calli formed from the cells, can develop into individual plants allow us to generate transgenic plants, the use of which has been recognized to be essential for the characterization of genes isolated. But, since the method for the regeneration of plants from cells of legume plants, to which azuki bean plants belong, has not been established, the preparation of transgenic legume plants has been considered to be difficult, or to be almost impossible. As I described in "Introduction" of Part III, I am thinking that azuki bean plants are most suitable plant materials for study of gibberellin promotion of stem elongation and, as mentioned above, I am also thinking that the use of transgenic plant is essential for the characterization of genes which I isolated. Therefore, I tried to establish the conditions that allow a few azuki bean cells to develop into individual plants.

Materials and Methods

Condition of culture — Seeds of azuki bean plants were sterilized by treating them with NaClO solution (2% active chlorine) for 1 h and the sterilized seeds were sown on solid medium that contained 1xMurashige and Skoog (MS) salt mixture (SIGMA), 1 % sucrose, 0.05 % MES-KOH buffer, pH5.7 and 0.3 % Phytagel (SIGMA). Sterilized seedlings were grown under continuous light at 25 °C for 6 days. Cross sections of epicotyls, 1 mm in thickness, were obtained from azuki bean epicotyls at sites between 5 and 20 mm below the node of the first leaves. These sections were put on solid basal medium (1xGamborg B5 salt mixture; SIGMA, 2 % glucose, 0.05 % MES-KOH buffer; pH 5.7, 100 mg/l *myo*-inositol, 20 mg/l thiamine-HCl, 1 mg/l pyridoxine-HCl, 1 mg/l nicotinic acid and 0.3 % Phytagel; SIGMA) that contained auxin (2,4-D) and cytokinin (BA) and were incubated under continuous light at 25 °C for one week for callus induction. Calli thus induced were transferred to solid basal medium that contained auxin (IAA) and cytokinin (*trans*-zeatin) and were incubated under continuous light at 25 °C for shoot induction. After five-weeks, the number of calli from which shoots were generated were counted.

Results

To find out the suitable hormonal conditions for azuki bean tissue to generate calli that have an ability to regenerate by transferring the calli on shoot-inducing medium, I examined the effects of auxin (2,4-D) at concentrations ranging from 50 to 2,000 μg/l and these of cytokinin (BA) at concentrations of 10 to 1,000 μg/l, first. Since these hormonal conditions which I employed allowed me to obtain well-growing calli, I transplanted the calli to various kinds of shoot inducing medium to let the calli to regenerate shoots. The well-growing calli that I obtained failed to regenerate shoots (data not shown). Therefore, I examined the effects of BA and 2,4-D at lower concentrations, which induced slowly growing calli, on shoots regeneration from calli.

Slowly growing calli were made by incubating epicotyl slices on solid basal media that contained 0-20 μg/l 2,4-D and 0-10 μg/l BA for one week and then were transferred to basal media that contained 0-80 μg/l IAA and 0-10 μg/l *trans*-zeatin to regenerate shoot. I examined how the concentrations of 2,4-D, BA, IAA and *trans*-zeatin in these media affected the development of calli into individual plants in these conditions (Table 4). I succeeded in shoots regeneration from plenty of these calli. The result indicated that the concentration of 2,4-D for shoot-inducible callus formation was strict and the suitable concentration was 10-20 μg/l (Table 4). The concentration of BA for shoot inducible callus formation was not so strict and the suitable concentration seemed lower than 1 μg/l (Table 4). In shoot inducing medium, the optimum concentration of IAA for shoot regeneration was 20-80 μg/l and the optimum concentration of *trans*-zeatin was 1-5 μg/l (Table 4).

These results showed that the optimum concentration of BA for shoot-inducible callus formation seemed lower than 1 μg/l. Therefore, I examined BA at 5 μg/l, 1 μg/l or lower for its effect on the formation of shoot-indusible calli (Table 5). In this experiment, induced calli were transplanted to shoot-inducing medium that contained 20 μg/l IAA and 1μg/l *trans*-zeatin. The result indicated that 10 μg/l 2,4-D and 0.1 μg/l BA were optimum for the induction of calli which had the ability to generate shoots on transplants to shoot inducing medium (Table

5). Shoots, thus regenerated, were normal and grew into whole plants (Fig. 30). These regenerated plants, which had been transferred to soil, bloomed and set seeds which could germinate (data not shown).

Discussion

It has been thought that preparations of transgenic plants are difficult in legume plants, because it was hard to regenerate their shoot from a few cells which were transformed. But, I desire to make transgenic azuki bean plants, though it is a kind of legumes, because azuki bean plants have an irreplaceable merit for study on elongation caused by GA_3 (Part III, Introduction). Therefore, I tried to regenerate shoots from calli induced from small pieces of azuki bean epicotyls, and succeeded in shoot regeneration. The results shows that the optimum concentration of cytokinin in shoot inducing medium and in callus inducing medium must be very low. For example, in shoot inducing medium, the concentration of *trans*-zeatin for shoot regeneration of *Arabidopsis*, that is generally used for experiments of transgenic plants, is at 2 mg/l (Kakimoto 1996), while the concentration of *trans*-zeatin for shoot regeneration of azuki bean plant must be 10 μg/l or lower (Table 4). Shoot regeneration of azuki bean plant was hard to occur in medium that contained *trans*-zeatin at 20 μg/l or higher (data not shown). This hyper sensitive nature of azuki bean plants to cytokinin may cause one of difficulties in seeking the conditions for shoot regeneration in azuki bean plants.

By using this method of shoot regeneration in azuki bean plants, now, I am trying to make kanamycin, an antibiotic,-resistant transgenic azuki bean plants from cells to which kanamycin-resistance gene was introduced.

General Discussion

The rearrangement of cortical MTs by GA_3 was originally demonstrated by electron microscopy (Shibaoka 1974). However, in this early study, the arrangement of MTs was observed only 6 h after the start of treatment with IAA plus GA_3, when the promotion by GA_3 of elongation was already evident. Thus, it has not been clear whether GA_3 changes the orientation of cortical MTs indirectly, by changing the rate of cell elongation, or directly, by a mechanism that does not involve a GA_3-induced change in the rate of cell elongation. In the present study, rearrangement of cortical MTs was evident 1 h after the start of treatment with IAA, after pretreatment with GA_3, when GA_3-induced promotion of IAA-induced elongation had not yet become evident. Thus observation indicates that the rearrangement of cortical MTs by GA_3 is not brought about as a result of GA_3-promoted elongation of cells. Recently, Sakiyama-Sogo and Shibaoka (1993) showed that GA_3 altered the arrangement of cortical MTs in decapitated epicotyl cuttings from GA_3-pretreated seedlings of the dwarf pea in which GA_3 did not alter the rate of epicotyl elongation and they suggested that GA_3 can alter the orientation of cortical MTs by a mechanism that does not involve a change in the rate of cell expansion. My present results lend further support to this view (Part I).

As reported previously (Shibaoka 1974), IAA alone did not cause cortical MTs to become oriented transversely to the cell axis (Fig. 5c, 6c, 7c, 8c). Since IAA has no effect on the arrangement of cortical MTs and since colchicine, a microtubule-disrupting agent, does not inhibit the IAA-induced elongation of epicotyl segments (Shibaoka 1972), it has been considered that cortical MTs are not involved in the IAA-induced elongation of azuki bean epicotyls (Shibaoka 1994). However, IAA has been reported to cause cortical MTs to become arranged transversely to the cell axis in tissues of many plant species, such as wheat coleoptiles (Volfová et al. 1977), oat mesocotyls (Iwata and Hogetsu 1989), maize coleoptiles (Bergfeld et al. 1988, Nick et al. 1990, Zandomeni and Schopfer 1993) and radish hypocotyls (Sakoda et al. 1992). The result reported by Mayumi and Shibaoka (1996) provide explanation for these IAA-induced increase in transverse MTs in those plant species. Mayumi and Shibaoka

(1996) suggested that IAA is necessary for reorientation of MTs from longitudinal to transverse but not for the reverse process and thus IAA kept the cyclic reorientation of cortical MTs. Treatment with basal medium, that caused the absence of IAA, inhibited the reorientation from longitudinal to transverse but not the reverse process. The accumulation of cells with longitudinal MTs after treatment without IAA resulted in the synchronized progression of the MT-reorientation cycle from longitudinal to transverse upon subsequent application of IAA. Therefore, the percentage of cells with transverse MTs increased in many plant species mentioned above. My present result showed that the percentage of cells with transverse MTs decreased and that of cells with longitudinal MTs increased during the period of treatment with basal medium (Fig. 5a, 6a, 7a) would be caused by the depletion of IAA. In the present study, I suggested the transcripts induced by GA_3 was involved in the reorientation of cortical MTs to transverse by GA_3. These transcripts induced by GA_3 may play a role in keeping the transverse MTs reoriented by IAA. I thinks that it is important to examine the locations of products of *GAUR* genes, that I isolated by differential screening (Part III), in cells in consideration of the position of cortical MTs.

GA_3 decreased the accumulation of mRNA for ACC-oxidase and the activity of ACC-oxidase in the segments of azuki bean epicotyls (Part II). I also found that GA_3 increased the accumulation of ACC in the segments of azuki bean epicotyls (Fig. 13). In recent years, it has been reported that GA_3 regulates the synthesis of SAM, a precursor of ACC in the ethylene biosynthesis pathway. GA_3 increases the accumulation of SAM and the activity of SAM synthetase in dwarf pea epicotyls (Mathur and Sachar 1991, Mathur et al. 1993), and the accumulation of *sam* mRNA, which encodes SAM synthetase, is increased by GA_3 in petunia corollas (Izhaki et al. 1996). Thus, it is probable that the increase in the accumulation of ACC by GA_3, which I found in the segments of azuki bean epicotyls, was caused by the increase in the accumulation of SAM by GA_3. SAM is not merely a precursor of ACC. It also acts as a methyl-group donor in various transmethylation reactions and as a propylamine group donor in the polyamine biosynthesis pathway. Thus there is a possibility that the gibberellin-induced accumulation of SAM facilitates the

gibberellin-induced stem elongation. Izhaki et al's result (1996) that *sam* gene is expressed at high level in elongating petunia stems and Boerjan et al's result (1994) that transgenic tobacco plants with reduced SAM synthetase activity show stunted phenotype may support this possibility. However, Boerjan et al. (1994) also reported that highly elevated SAM synthetase activity also led to the inhibition of stem elongation. SAM seems to be necessary for stem elongation but excess SAM seems to cause deleterious effect on stem elongation. If excess SAM causes the inhibition of stem elongation by increasing the amount of ACC and, therefore, ethylene evolution, gibberellin-induced decrease in the expression of ACC-oxidase may partially compensate the inhibition of stem elongation which is caused by gibberellin-induced increase in the amount of SAM. Although gibberellin-induced decreases in the accumulation of *AB-ACO* mRNA and in the activity of ACC-oxidase do not seem to be responsible for gibberellin-induced stem elongation, they undoubtedly contribute to gibberellin-induced stem elongation. If the elongation of GA_3 is involved in the accumulation of SAM, there will be a possibility that transmethylation reactions take part in GA_3-induced stem elongation. However, there is no evidence indicating relationship GA_3-induced elongation with transmethylation reactions. The investigation of the relationship *GAUR2* gene, that had the sequence similarity to DNA-3-methyladenineglycosidase (Fig. 26), with 3-methyladenine might seem to provide hints on the relationship GA_3-induced elongation with transmethylation reactions.

I am examining how to make transgenic plants in azuki bean plants with an intention to obtain direct evidences for indicating the involvement of *GAUR* genes in GA_3-induced stem elongation. I succeeded in shoots regeneration from calli in azuki bean plants (Part IV), and now I am trying to make transgenic plants of azuki bean. Transgenic azuki bean plants will help me to examine the relationship between GA_3-induced elongation and *GAUR* genes.

Acknowledgement

I thank professor Hiroh Shibaoka for his kind guidance and for continuous patient encouragements. Thanks are also due to all of the members of the Laboratory of Cell Physiology. I thank Dr. Hitoshi Mori (Nagoya University, Nagoya, Japan) for technical advice on measurement of ethylene. This study was supported by Research Fellowships of the Japan Society for the promotion of Science for Young Scientists (No. 2098).

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Table 1. Inhibition by actinomycin D of the elongation of segments of azuki bean epicotyls caused by GA_3, IAA, or IAA + GA_3.

	Elongation of segments (mm) [a]	
	- actinomycin D	+ actinomycin D (30 μM)
Basal medium	0.8±0.2	0.7±0.2
+GA_3 100 μM	0.9±0.1	0.8±0.2
+IAA 100 μM	3.1±0.4	1.5±0.2
+IAA + GA_3	5.6±0.5	1.7±0.2

a Average values for 10 segments with standard deviations.

Table 2. Promotion by pretreatment with GA3 of the elongation of segments of azuki bean epicotyls caused by IAA and inhibition by actinomycin D of the promotion by GA3 of the elongation.[a]

Conc. of actinomycin D (μM)	Elongation of segments (mm)[b]	
	- GA3 pretreatment	+ GA3 pretreatment
0	4.0±0.4	7.4±0.7
3	4.1±0.5	6.0±0.7
10	4.4±0.5	5.1±0.5
30	4.0±0.5	4.6±0.3

a Segments were pretreated with basal medium (- GA3) or basal medium + 100 μM GA3 (+ GA3) for 1 h and were then incubated in basal medium + 100 μM IAA for 14 h. Actinomycin D was applied during the pretreatment with GA3.

b Average values for 10 segments with standard deviations.

Table 3 Inhibition by GA_3 of increase in the activity of ACC-oxidase

		ACC-oxidase activity[a]	Increase in the activity[a]	Inhibition of the increase in the activity (%)
Initial		74.7±5.4	–	–
6h	Basal medium	160.9±19.5	86.2	–
	+GA_3	128.9±1.4	54.2	37
	+IAA	125.0±10.8	50.3	42
	+IAA+GA_3	91.1±3.2	16.4	81
8h	Basal medium	236.4±7.1	161.7	–
	+GA_3	187.1±7.0	112.4	30
	+IAA	237.2±21.1	162.5	0
	+IAA+GA_3	101.0±15.8	26.3	82

[a] C_2H_4 produced, nl / hr / 25 segments. Averages for three sets of twenty-five segments with standard errors.

Table 4 The effect of 2,4-D and BA in callus inducing medium and IAA and zeatin in shoot inducing medium on shoots regeneration from calli in sections of azuki bean epicotyls.

2,4-D[a]		0					2					5					10					20				
BA[b] \ IAA[c]		0	10	20	40	80	0	10	20	40	80	0	10	20	40	80	0	10	20	40	80	0	10	20	40	80
zeatin[d]	0			+			+										+					+			+	
	1						+							+	+						+			+	+	+
0	2							+									+		++		+	+		+	+	+
	5														+	+	+		+		+	+		+	+	+
	10						+			+				+					++			+				
	0																									
	1																		+	+	+					
1	2										+								+		+					++
	5									+	+			+	+	+			+							
	10					+			+							+			+	+	+			+	+	
	0														+					+	+					+
	1																		+	+	++	+				
2	2												+				+									
	5																		+		+					
	10														+			+	+	+		+				
	0																									+
	1				+													+			+				+	+
5	2														+	+						+				+
	5												+						+							
	10										+		+									+				
	0																									
	1	+													+	+	+				+				+	
10	2												+									+	+			
	5			+																	+					
	10																		+						+	

[a] The concentration of 2,4-D in callus inducing medium (μg/l)
[b] The concentration of BA in callus inducing medium (μg/l)
[c] The concentration of IAA in shoot inducing medium (μg/l)
[d] The concentration of *trans*-zeatin in shoot inducing medium (μg/l)

Two calli were tested in each medium and the plus mark shows callus shoot was regenerated from which.

Table 5 The effect of 2,4-D and BA in callus inducing medium on shoots regeneration from calli in sections of azuki bean epicotyls.

BA[b] \ 2,4-D[a]	5	10	20	50
0		++	++	
0.1	++	+++	++	
0.5		+++	+	
1				
5	+			

[a] The concentration of 2,4-D in callus inducing medium (μg/l)
[b] The concentration of BA in callus inducing medium (μg/l)

Three calli were tested in each medium and the plus mark shows callus shoot was regenerated from which.

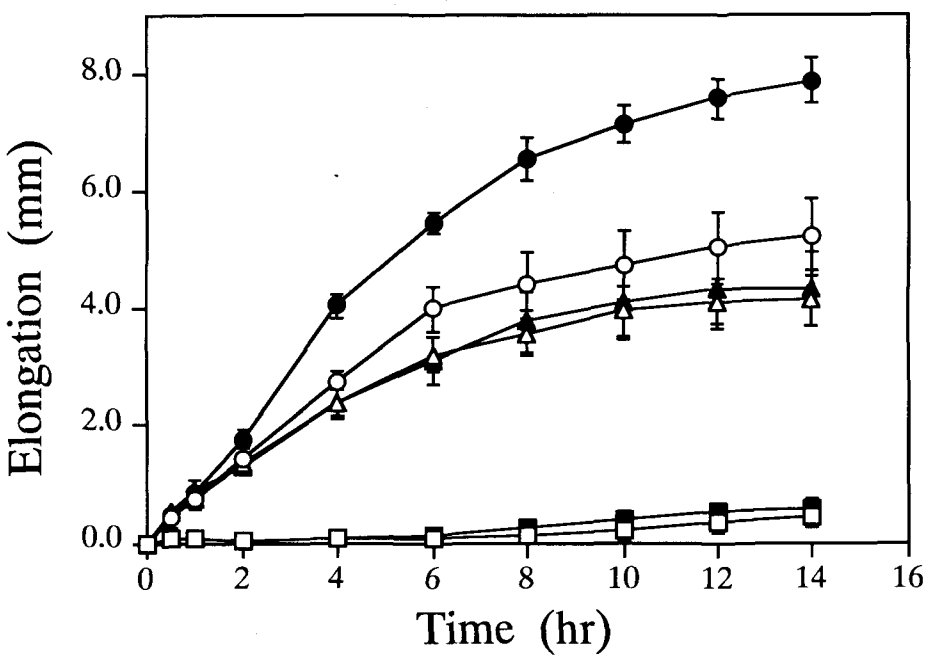


Fig. 1 Effects of pretreatment with GA3 (100 μM) on the time course of changes in length of segments of azuki bean epicotyls caused by subsequent incubation with IAA (100 μM) and the effects of actinomycin D (30 μM) on the promotion by GA3 of IAA-induced elongation or on IAA-induced elongation in the absence of pretreatment with GA3. -□-, Pretreatment with basal medium and subsequent incubation in basal medium; -■-, pretreatment with GA3 (100 μM) and subsequent incubation in basal medium; -○-, pretreatment with basal medium and subsequent incubation with IAA (100 μM); -●-, pretreatment with GA3 (100 μM) and subsequent incubation with IAA (100 μM); -△-, pretreatment with actinomycin D (30 μM) and subsequent incubation with IAA (100 μM); -▲-, pretreatment with GA3 (100 μM) plus actinomycin D (30 μM) and subsequent incubation with IAA (100 μM). Averages for 10 segments with standard deviations (bar).

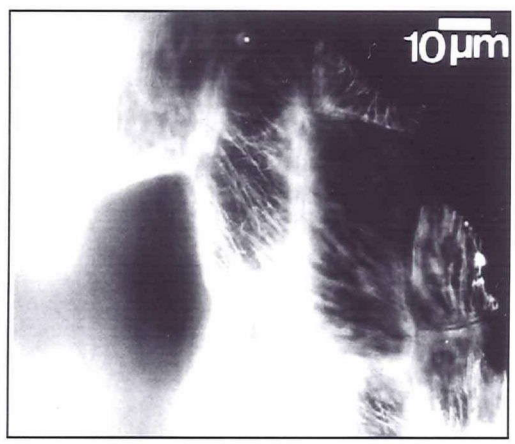


Fig. 2 Immunofluorescence image of arrays of cortical MTs adjacent to the outer tangential walls of epidermal cells of an untreated azuki bean seedling. Bar=10 μm.

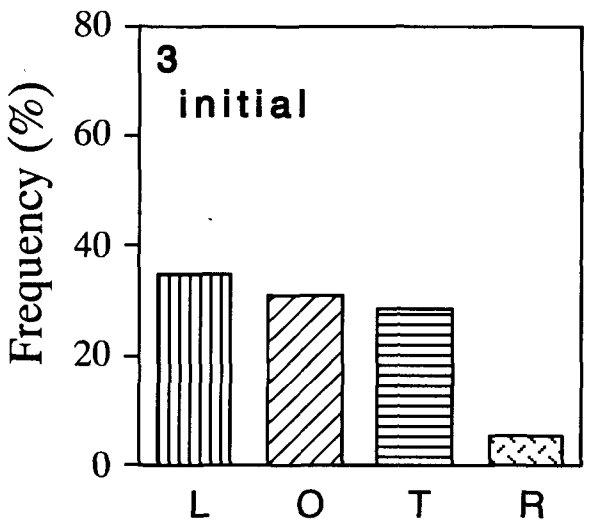


Fig. 3 The arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells from untreated azuki bean seedlings. The histogram shows the percentages of cells with MTs within 0-30° (longitudinal, L), 30-60° (oblique, O) and 60-90° (transverse, T) of the cell axis. The percentage of cells with MTs in a variety of directions (random, R) is also indicated. n=325.

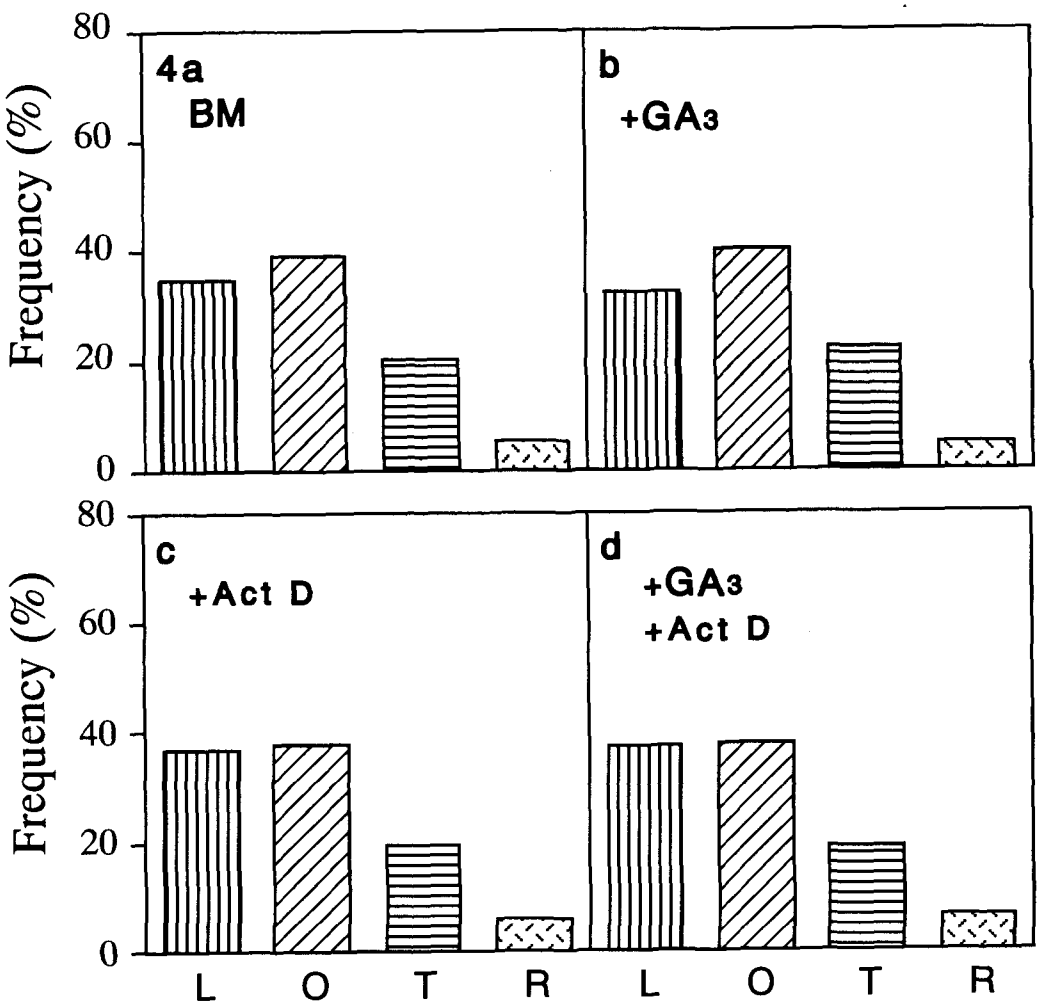
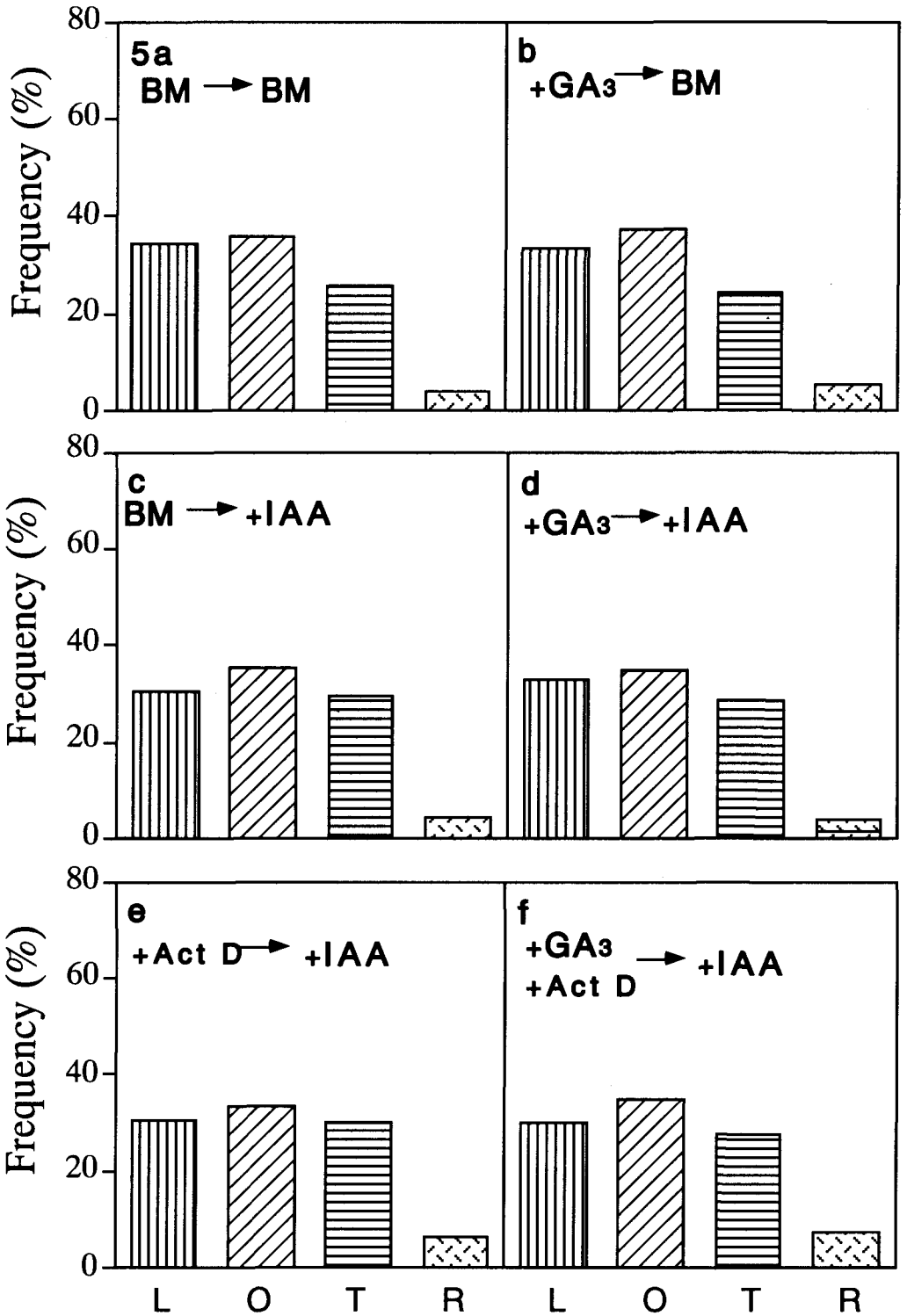


Fig. 4 The effect of a 1-h pretreatment with basal medium, with basal medium plus 100 μM GA3, with basal medium plus 30 μM actinomycin D or with basal medium plus GA3 and actinomycin D on the arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells of segments of azuki bean epicotyls. a, Treatment with basal medium (BM, n=252); b, treatment with basal medium plus GA3 (+ GA3, n=356); c, treatment with basal medium plus actinomycin D (+ Act D, n=322); d, treatment with basal medium plus GA3 and actinomycin D (+ GA3 + Act D, n=294). See legend to Fig. 3 for further details.



Figs. 5 The arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells of segments of azuki bean epicotyls after subsequent incubation for 0.5 h. a, pretreatment with basal medium and subsequent incubation with basal medium (n=313); b, pretreatment with basal medium plus GA3 (100 μM) and subsequent incubation with basal medium (n=417); c, pretreatment with basal medium and subsequent incubation with basal medium plus IAA (100 μM) (n=405); d, pretreatment with basal medium plus GA3 and subsequent incubation with basal medium plus IAA (n=392); e, pretreatment with basal medium plus actinomycin D and subsequent incubation with basal medium plus IAA (n=308); f, pretreatment with basal medium plus GA3 and actinomycin D and subsequent incubation with basal medium plus IAA (n=351). See legend to Fig. 3 for further details.

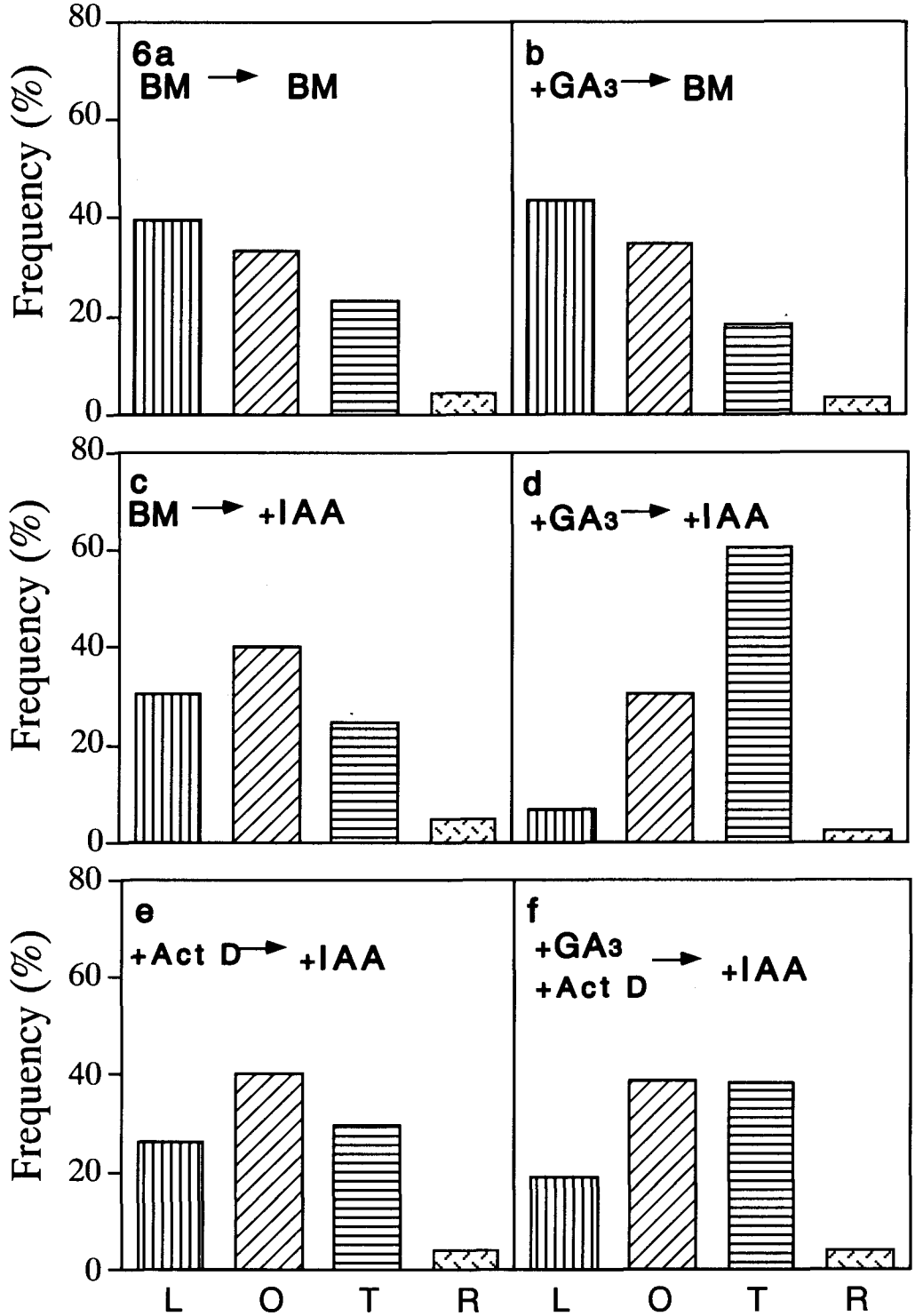


Fig. 6 The arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells of segments of azuki bean epicotyls after subsequent incubation for 1.0 h. a, pretreatment with basal medium and subsequent incubation with basal medium (n=404); b, pretreatment with basal medium plus GA3 and subsequent incubation with basal medium (n=376); c, pretreatment with basal medium and subsequent incubation with basal medium plus IAA (n=366); d, pretreatment with basal medium plus GA3 and subsequent incubation with basal medium plus IAA (n=350); e, pretreatment with basal medium plus actinomycin D and subsequent incubation with basal medium plus IAA (n=365;); f, pretreatment with basal medium plus GA3 and actinomycin D and subsequent incubation with basal medium plus IAA (n=319). See legend to Fig. 3 for further details.

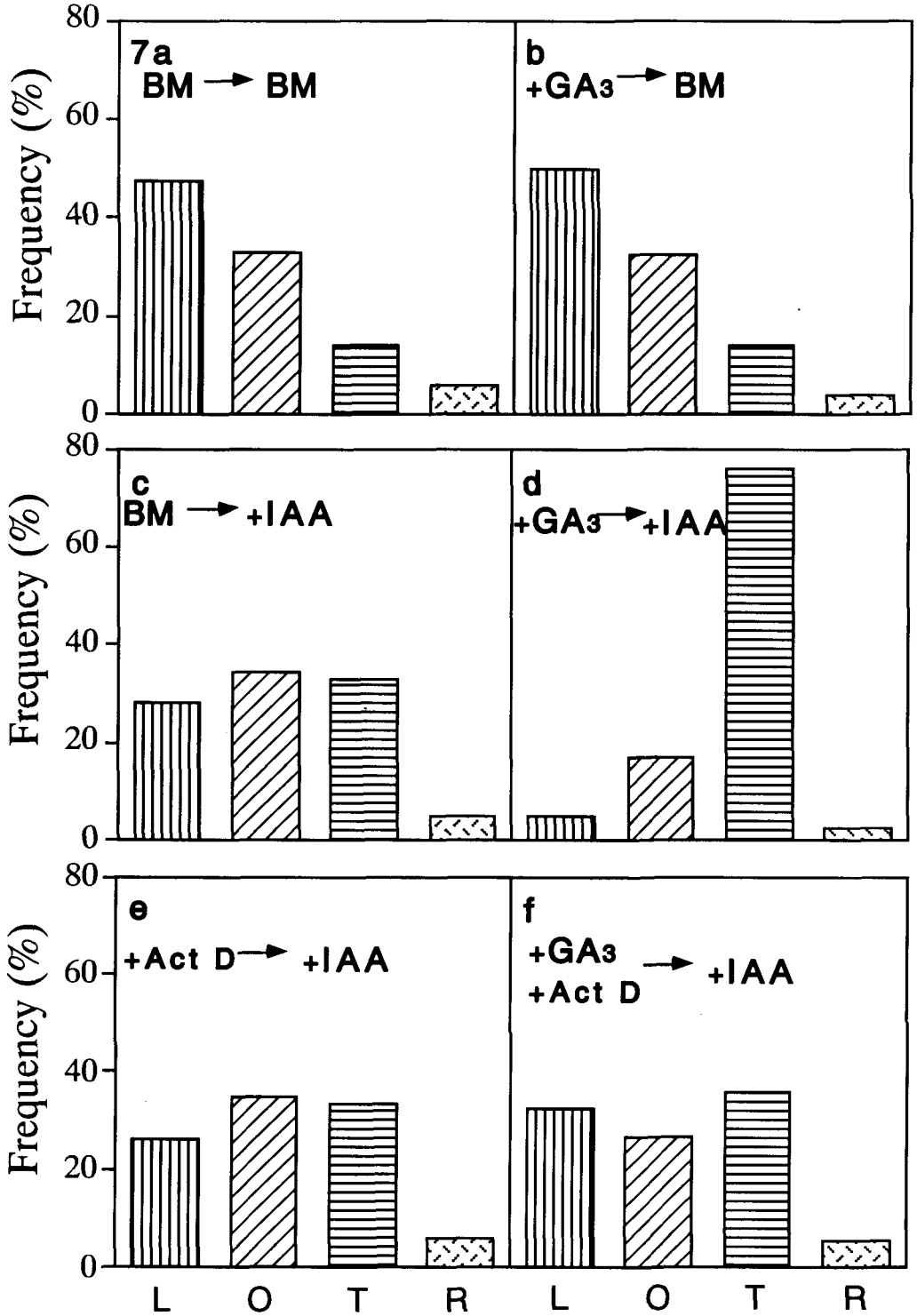


Fig. 7 The arrangement of cortical MTs adjacent to the outer tangential walls of epidermal cells of segments of azuki bean epicotyls after subsequent incubation for 2.0 h. a, pretreatment with basal medium and subsequent incubation with basal medium (n=267); b, pretreatment with basal medium plus GA3 and subsequent incubation with basal medium (n=271); c, pretreatment with basal medium and subsequent incubation with basal medium plus IAA (n=257); d, pretreatment with basal medium plus GA3 and subsequent incubation with basal medium plus IAA (n=334); e, pretreatment with basal medium plus actinomycin D and subsequent incubation with basal medium plus IAA (n=280); f, pretreatment with basal medium plus GA3 and actinomycin D and subsequent incubation with basal medium plus IAA (n=259). See legend to Fig. 3 for further details.

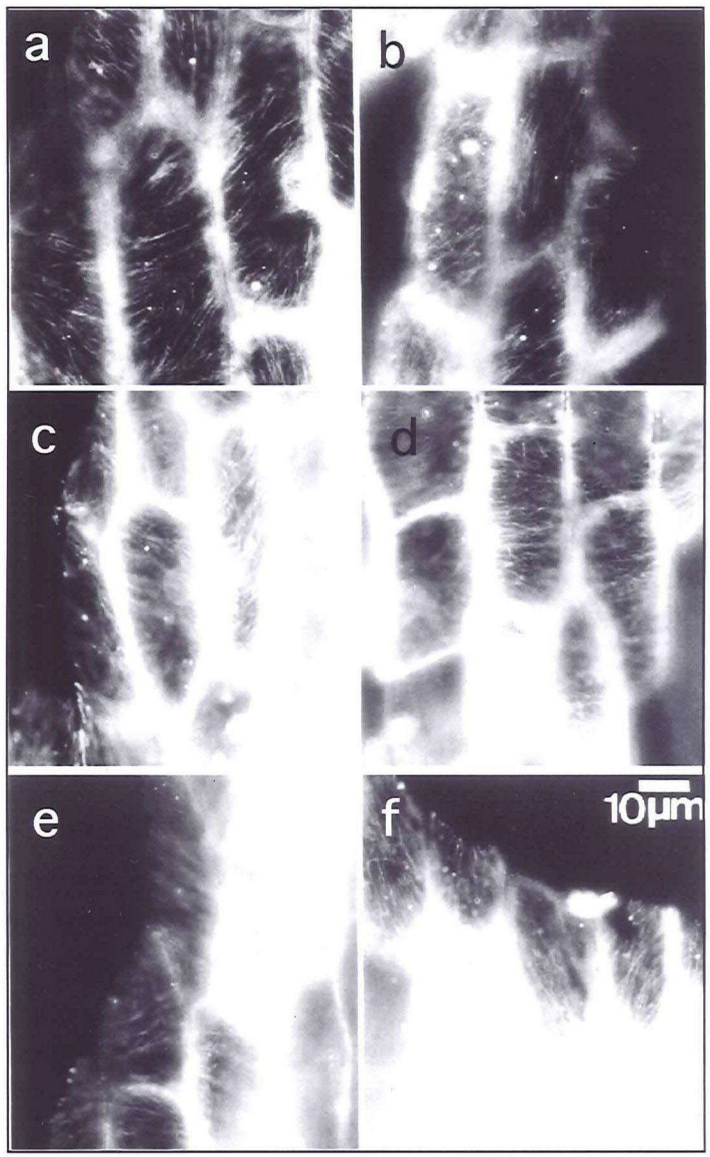


Fig. 8 Immunofluorescence images of arrays of cortical MTs adjacent to the outer tangential walls of epidermal cells of azuki bean seedlings after pretreatment and 2.0-h subsequent incubation. a, Pretreatment with basal medium and subsequent incubation with basal medium; b, pretreatment with basal medium plus GA_3 and subsequent incubation with basal medium; c, pretreatment with basal medium and subsequent incubation with basal medium plus IAA; d, pretreatment with basal medium plus GA_3 and subsequent incubation with basal medium plus IAA; e, pretreatment with basal medium plus actinomycin D and subsequent incubation with basal medium plus IAA; f, pretreatment with basal medium plus GA_3 and actinomycin D and subsequent incubation with basal medium plus IAA. Bar=10 μm.

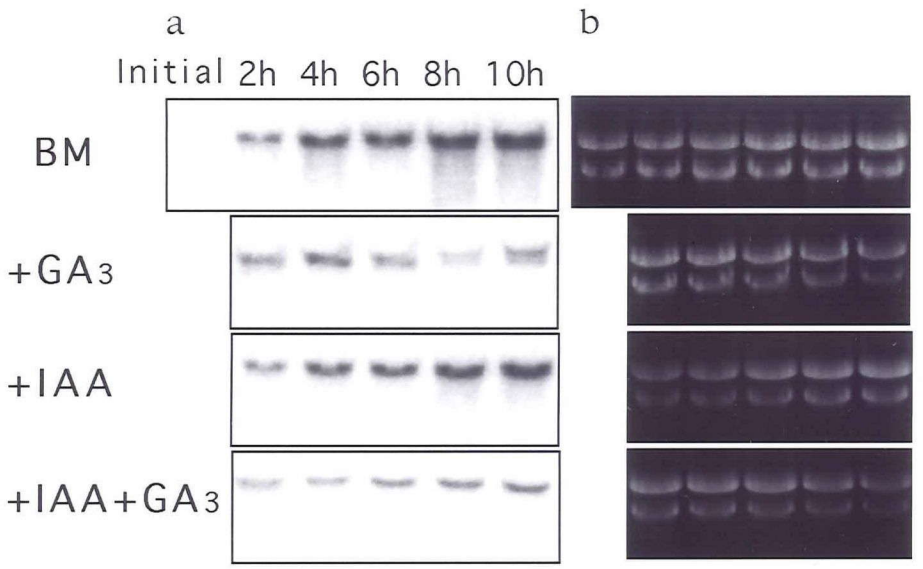


Fig. 9 Effects of GA_3, IAA and IAA+GA_3 on time course of changes in the accumulation of mRNA of the GA_3 down-regulated gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA_3, treated with basal medium that contained GA_3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA_3, treated with basal medium that contained IAA and GA_3. a, Northern blot analysis using α-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

```
1         10         20         30         40         50         60         70         80
CTCAAACATTAAGATAAGGGAACAAGAGATTGAAGAAGTGAGAGATGGAGAACTTCCCAGTGATCAACCTTGAGAATATC
                                              M  E  N  F  P  V  I  N  L  E  N  I

81        90         100        110        120        130        140        150        160
AATGGTGAGGAGAGAAAGGCTGTCCTAGAGAAAATCGAAGATGCTTGTGAAAACTGGGGATTTTTTGAGTTGGTAAATCA
N  G  E  E  R  K  A  V  L  E  K  I  E  D  A  C  E  N  W  G  F  F  E  L  V  N  H

161       170        180        190        200        210        220        230        240
TGGAATACCCCTTGAACTTTTGGATACTGTGGAAAGGTTAACGAAAGAGCATTATAGGAAATGCATGGAGCAAAGGTTCA
 G  I  P  L  E  L  L  D  T  V  E  R  L  T  K  E  H  Y  R  K  C  M  E  Q  R  F  K

241       250        260        270        280        290        300        310        320
AGGAGGCAGTGGCAAGCAAGGGATTGGAGGGTGTCCAAGGTGAAATAAAGGACATGGACTGGGAGAGCACCTTCTTCTTG
  E  A  V  A  S  K  G  L  E  G  V  Q  G  E  I  K  D  M  D  W  E  S  T  F  F  L

321       330        340        350        360        370        380        390        400
CGCCATCTTCCACATTCAAACATCTCTGAAATTCCTGATCTCAGTGAAGAGTACAGGAAGGCAATGAAGGACTTTGCACA
R  H  L  P  H  S  N  I  S  E  I  P  D  L  S  E  E  Y  R  K  A  M  K  D  F  A  Q

401       410        420        430        440        450        460        470        480
AAAATTGGAGAAACTTGCAGAGGAGCTGCTTGACCTTTTGTGTGAAAATCTTGGATTAGAGAAAGGGTACCTGAAGAAAG
 K  L  E  K  L  A  E  E  L  L  D  L  L  C  E  N  L  G  L  E  K  G  Y  L  K  K  A

481       490        500        510        520        530        540        550        560
CCTTCTATGGATCAAAGGGTCCAAATTTTGGCACAAAGGTAGCAAACTACCCTCCATGTCCGAAGCCAGAGCTGGTGAAG
  F  Y  G  S  K  G  P  N  F  G  T  K  V  A  N  Y  P  P  C  P  K  P  E  L  V  K

561       570        580        590        600        610        620        630        640
GGTCTTCGTGCTCATACAGATGCAGGTGGGATTATCCTTCTCTTGCAAGATGACAAGGTCAGTGGCCTGCAGCTTCTCAA
G  L  R  A  H  T  D  A  G  G  I  I  L  L  L  Q  D  D  K  V  S  G  L  Q  L  L  K

641       650        660        670        680        690        700        710        720
AGATGGCCAGTGGGTGGATGTGCCTCCAATGCGCCACTCCATTGTTGTTAACCTTGGTGACCAGCTTGAGGTAATTACCA
 D  G  Q  W  V  D  V  P  P  M  R  H  S  I  V  V  N  L  G  D  Q  L  E  V  I  T  N

721       730        740        750        760        770        780        790        800
ATGGGAAATACAAAAGTGTGGAGCACCGTGTGATTGCTAGAACTGATGGGACTAGAATGTCCATAGCCTCATTCTACAAC
  G  K  Y  K  S  V  E  H  R  V  I  A  R  T  D  G  T  R  M  S  I  A  S  F  Y  N

801       810        820        830        840        850        860        870        880
CCTGCTGGTGATGCTGTTATCTATCCTGCACCAGCATTGCTGGAGAGAAAAACACAAGTGATAGACAAAGTATACCCAAA
P  A  G  D  A  V  I  Y  P  A  P  A  L  L  E  R  K  T  Q  V  I  D  K  V  Y  P  K

881       890        900        910        920        930        940        950        960
ATTTGTGTTTGAGGATTACATGAGGCTCTACGCTACACTGAAGTTCCAACCAAAGGAACCAAGATTTGAAGCTATGAAAG
 F  V  F  E  D  Y  M  R  L  Y  A  T  L  K  F  Q  P  K  E  P  R  F  E  A  M  K  G

961       970        980        990        1000       1010       1020       1030       1040
GCGTGAACACAGTTTAGTTAGTTCCAACCAAACAACTTAATTGGAGCTGTGCTATACTATACTGCTAGAAGAAATTTTAT
  V  N  T  V  *

1041      1050       1060       1070       1080       1090       1100       1110       1120
TGATTTGGAAGAAGTGAGGAAATAGAATGCAATTAAGCAAGAAATAATGGTTGTTTAATCATTCAATTTCTTAATACAGT
1121      1130       1140       1150       1160       1170       1180       1190       1200
GGATTGTTTATTTGTACTTCAGAATTGTTGAAATAATATGCAACATTTCTACTCGTTCTATCTTAAAAAAAAAAAAAAAAA
```

Fig. 10 The nucleotide sequence and deduced amino acid sequence of a cDNA the accumulation of mRNA corresponding to which is decreased by GA_3. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

```
          1                              30 31                                60 61
Pch313    MENFPIINLEGLNGEGRKATMEKIKDACEN WGFFELVSHGIPTEFLDTVERLTKEHYRQC LEQRFKELVASKGLE  75
pGEFE     MESFPVINMEKLNGEERAATMEKIKDACEN WGFFELLNHGIPYELLDTVEKMTKEHYRKC MEQRFKEMVASKGLE  75
pTOM5     MENFPIINLENLNGDERAKTMEMIKDACEN WGFFELVNHGIPHEVMDTVEKLTKGHYKKC MEQRFKELVASKGLE  75
pTOM13                       MEMIKDACEN WGFFELVNHGIPHEVMDTVEKMTKGHYKKC MEQRFKELVASKGLE  55
AB-ACO    MENFPVINLENINGEERKAVLEKIEDACEN WGFFELVNHGIPLELLDTVERLTKEHYRKC MEQRFKEAVASKGLE  75

          76             90 91                            120 121                   150
Pch313    AVKTEVNDMDWESTF YLRHLPKSNISEVPDLEDQYRNVMKEFALK LEKLAEQLLDLLCENLGLEQGYLKKAFYGT 150
pGEFE     GVEVEVEDLDWESTF FLKHLPESNISQVPDLQDEYRKVMKEFAAK LEKLAEELLDLLSENLGLEKGYLKKAFYGS 150
pTOM5     AVQAEVTDLDWESTF FLRHLPTSNISQVPDLDEEYREVMRDFAKR LEKLAEELLDLLCENLGLEKGYLKNAFYGS 150
pTOM13    AVQAEVTDLDWESTF FLRHLPTSNISQVPDLDEEYREVMRDFAKR LEKLAEELLDLLCENLGLEKGYLKNAFYGS 130
AB-ACO    GVQGEIKDMDWESTF FLRHLPHSNISEIPDLSEEYRKAMKDFAQK LEKLAEELLDLLCENLGLEKGYLKKAFYGS 150

          151                            180 181                           210 211
Pch313    NGPTFGTKVSNYPPCPKPELIKGLRAHTDA GGLILLFQDDKVSGLQLLKDGQWIDVPPMR HSIVINLGDQLEVIT 225
pGEFE     KGPTFGTKVSNYPPCPKPDLIKGLRAHTDA GGLILLFQDDKVSGLQLLKDGKWVDVPPMH HSIVINLGDQLEVIT 225
pTOM5     KGPNFGTKVSNYPPCPKPDLIKGLRAHTDA GGIILLFQDDKVSGLQLLKDEQWIDVPPMR HSIVVNLGDQLEVIT 225
pTOM13    KGPNFGTKVSNYPPCPKPDLIKGLRAHTDA GGIILLFQDDKVSGLQLLKDEQWIDVPPMR HSIVVNLGDQLEVIT 205
AB-ACO    KGPNFGTKVANYPPCPKPELVKGLRAHTDA GGIILLLQDDKVSGLQLLKDGQWVDVPPMR HSIVVNLGDQLEVIT 225

          226            240 241                           270 271                   300
Pch313    NGKYKSVEHRVIAQT DGTRMSIASFYNPGSDAVIYPAPTLVEKEA EEKNQVYPKFVFEDYMKLYAGLKFQPKEPR 300
pGEFE     NGKYKSIEHRVIAQS DGTRMSIASFYNPGSDAVIYPAPALLEKET EEK-QVYPKFVFEDYMKLYSGLKFQAKEPR 299
pTOM5     NGKYKSVMHRVIAQT DGTRMSLASFYNPGNDAVIYPAPSLIE--- -ESKQVYPKFVFDDYMKLYAGLKFQPKEPR 296
pTOM13    NGKYKSVLHRVIAQT DGTRMSLASFYNPGSDAVIYPAKTLVEKEA EESTQVYPKFVFDDYMKLYAGLKFQAKEPR 280
AB-ACO    NGKYKSVEHRVIART DGTRMSIASFYNPAGDAVIYPAPALLERKT QVIDKVYPKFVFEDYMRLYATLKFQPKEPR 300

           301           315 316
Pch313    FEAMKAVETNIS-LG PIATA    319
pGEFE     FEAMKAVEANVT-LD PIRTA    318
pTOM5     FEAMKAMEANVELVD QIASA    316
pTOM13    FEAMKAMES-----D PIASA    295
AB-ACO    FEAMKGVNTV----- -----    310
```

Fig. 11 Comparison of the deduced amino acid sequences of ACC-oxidases and ACC-oxidase homologs in plants. Pch313 from peach (Callahan et al. 1992); pGEFE-1 from geranium (Wang et al. 1994); pTOM5 and pTOM13 from tomato (Holdsworth et al. 1987); AB-ACO from azuki bean epicotyls. Optimization of alignment was achieved with the ClustalW program (Human Genome Center, Baylor College of Medicine). Dashes indicate gaps introduced to maximize alignment. AB-ACO sequence and identical amino acids of Pch313, pGEFE-1, pTOM5 and pTOM13 with those of AB-ACO are shaded.

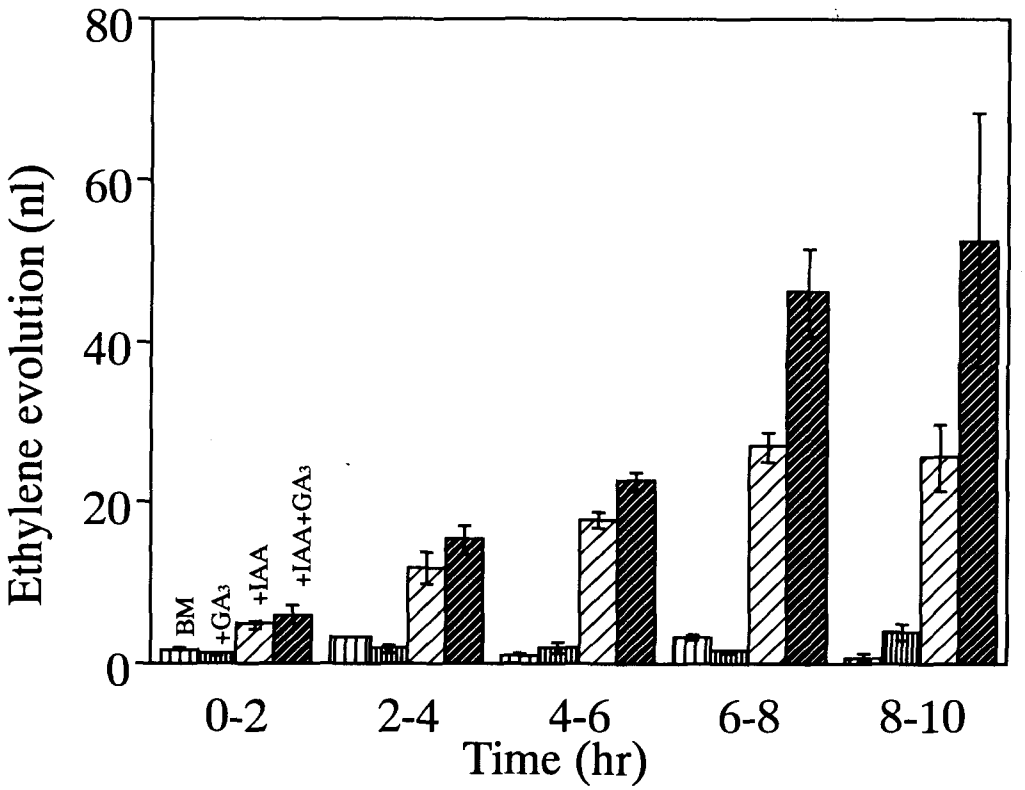


Fig. 12 Effects of GA3, IAA and IAA+GA3 on the rate of ethylene evolution in segments of azuki bean epicotyls. ▥, segments treated with basal medium;▩, segments treated with basal medium that contained GA3 (100 μM);▨, segments treated with basal medium that contained IAA (100 μM);▩, segments treated with basal medium that contained IAA and GA3. Ethylene evolution, nl / 2 h / 25 segments. Averages for three sets of twenty-five segments with standard errors (bar).

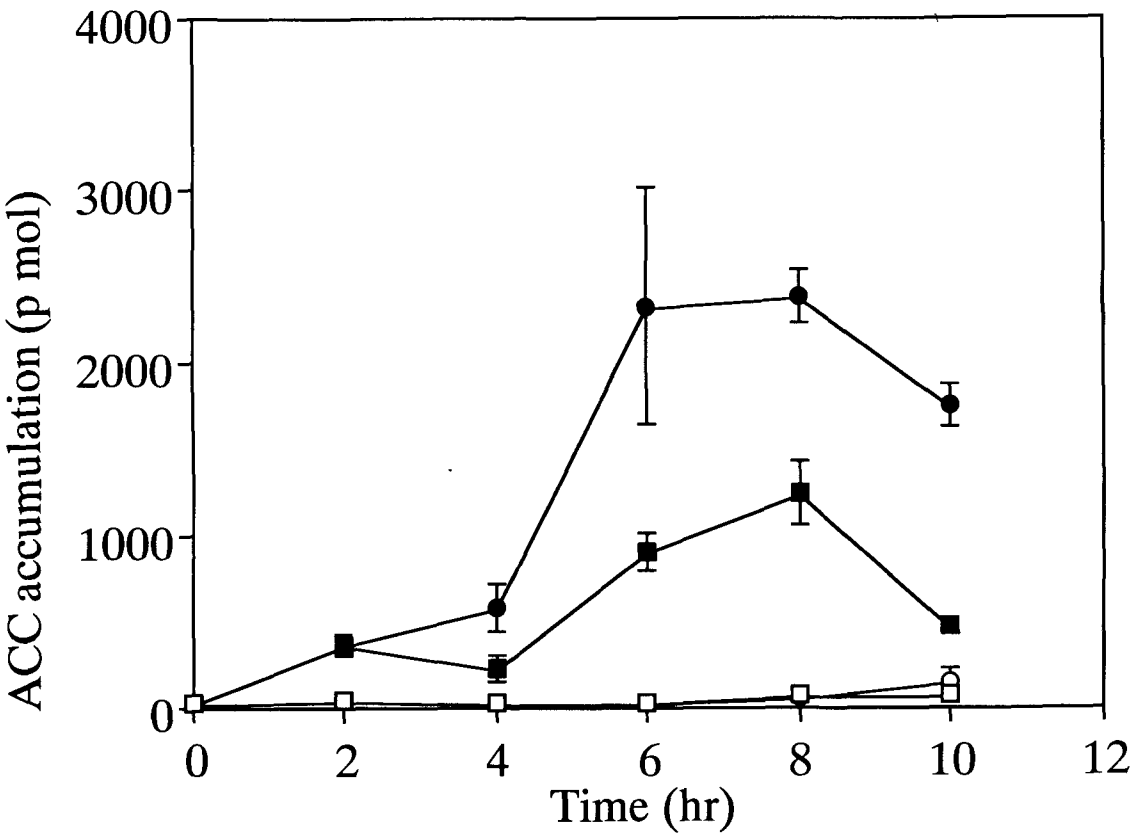


Fig. 13 Effects of GA3, IAA and IAA+GA3 on ACC accumulation in the segments of azuki bean epicotyls.-□-; segments treated with basal medium; -○-; segments treated with basal medium that contained GA3 (100 μM); -■-; segments treated with basal medium that contained IAA (100 μM); -●-; segments treated with basal medium that contained IAA and GA3. ACC accumulation, pmol / 25 segments. Averages for three sets of twenty-five segments with standard errors (bar).

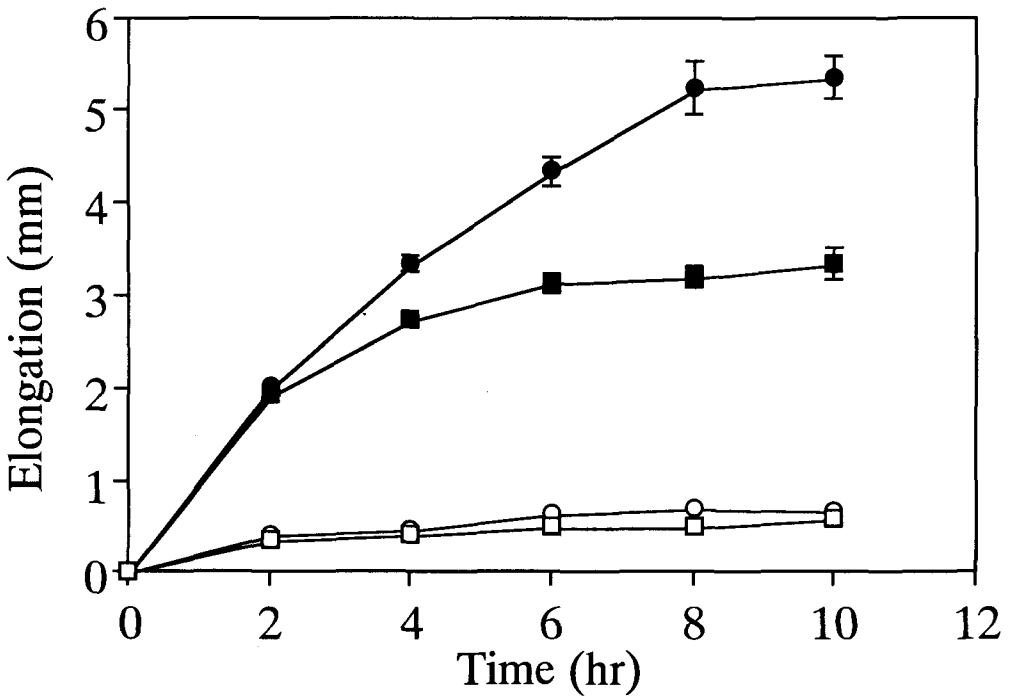


Fig. 14 Effects of GA3, IAA and IAA+GA3 on the time course changes in length of the segments of azuki bean epicotyls. -□-, segments treated with basal medium;-○-, segments treated with basal medium that contained GA3 (100 μM); -■- segments treated with basal medium that contained IAA (100 μM); -●- segments treated with basal medium that contained IAA and GA3. Averages for twenty-five segments with standard errors (bar).

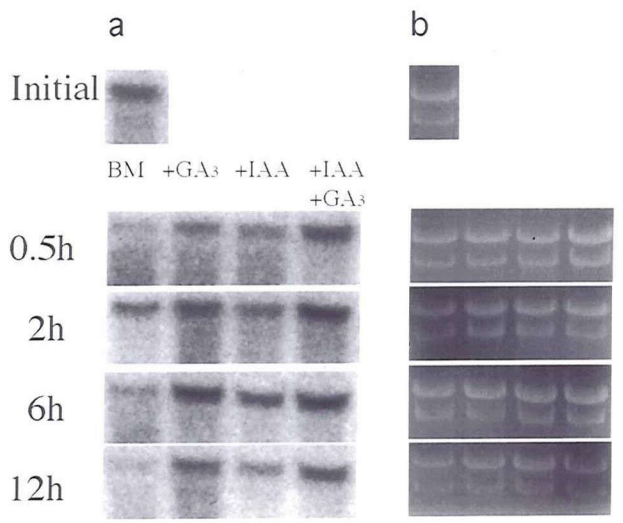


Fig. 15 Effects of GA3, IAA and IAA+GA3 on time course of changes in the accumulation of mRNA of *GAUR1* gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA3, treated with basal medium that contained GA3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA3, treated with basal medium that contained IAA and GA3. a, Northern blot analysis using α-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

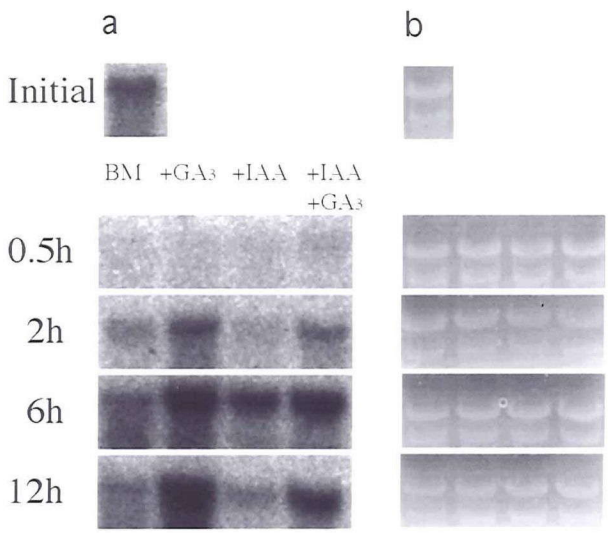


Fig. 16 Effects of GA_3, IAA and IAA+GA_3 on time course of changes in the accumulation of mRNA of *GAUR2* gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA_3, treated with basal medium that contained GA_3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA_3, treated with basal medium that contained IAA and GA_3. a, Northern blot analysis using α-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

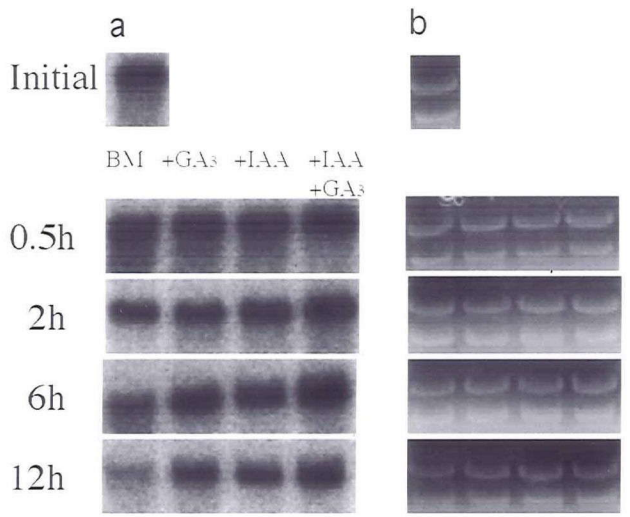


Fig. 17 Effects of GA_3, IAA and IAA+GA_3 on time course of changes in the accumulation of mRNA of *GAUR3* gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA_3, treated with basal medium that contained GA_3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA_3, treated with basal medium that contained IAA and GA_3. a, Northern blot analysis using a-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

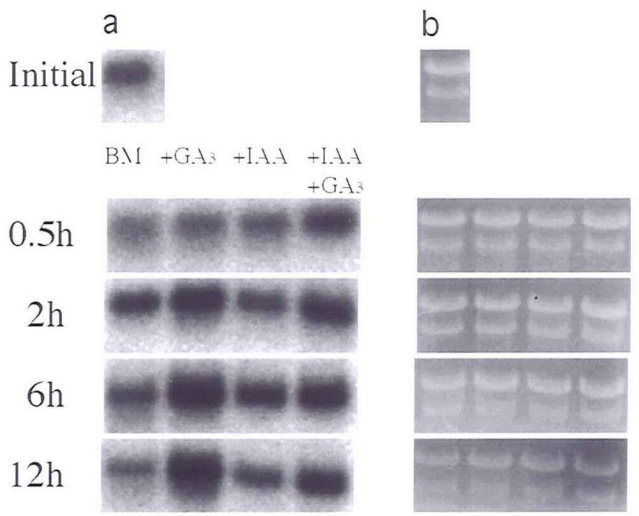


Fig. 18 Effects of GA_3, IAA and IAA+GA_3 on time course of changes in the accumulation of mRNA of *GAUR4* gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA_3, treated with basal medium that contained GA_3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA_3, treated with basal medium that contained IAA and GA_3. a, Northern blot analysis using α-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

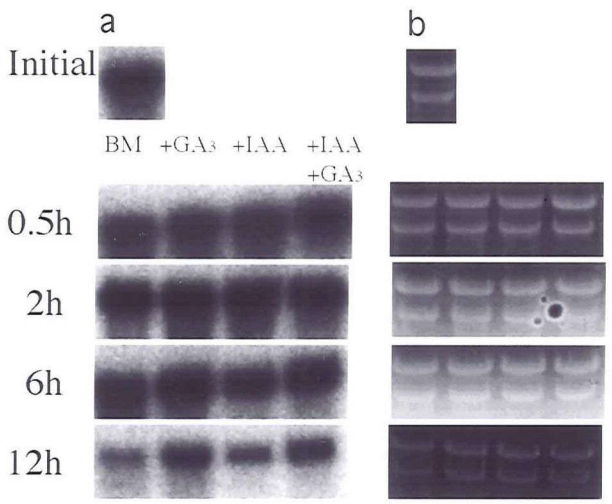


Fig. 19 Effects of GA_3, IAA and IAA+GA_3 on time course of changes in the accumulation of mRNA of *GAUR5* gene in segments of azuki bean epicotyls. Initial, before treatment; BM, treated with basal medium; +GA_3, treated with basal medium that contained GA_3 (100 μM); +IAA, treated with basal medium that contained IAA (100 μM); +IAA+GA_3, treated with basal medium that contained IAA and GA_3. a, Northern blot analysis using α-^{32}P dCTP labeled probe. b, Staining with ethidium bromide as a control for equal loading.

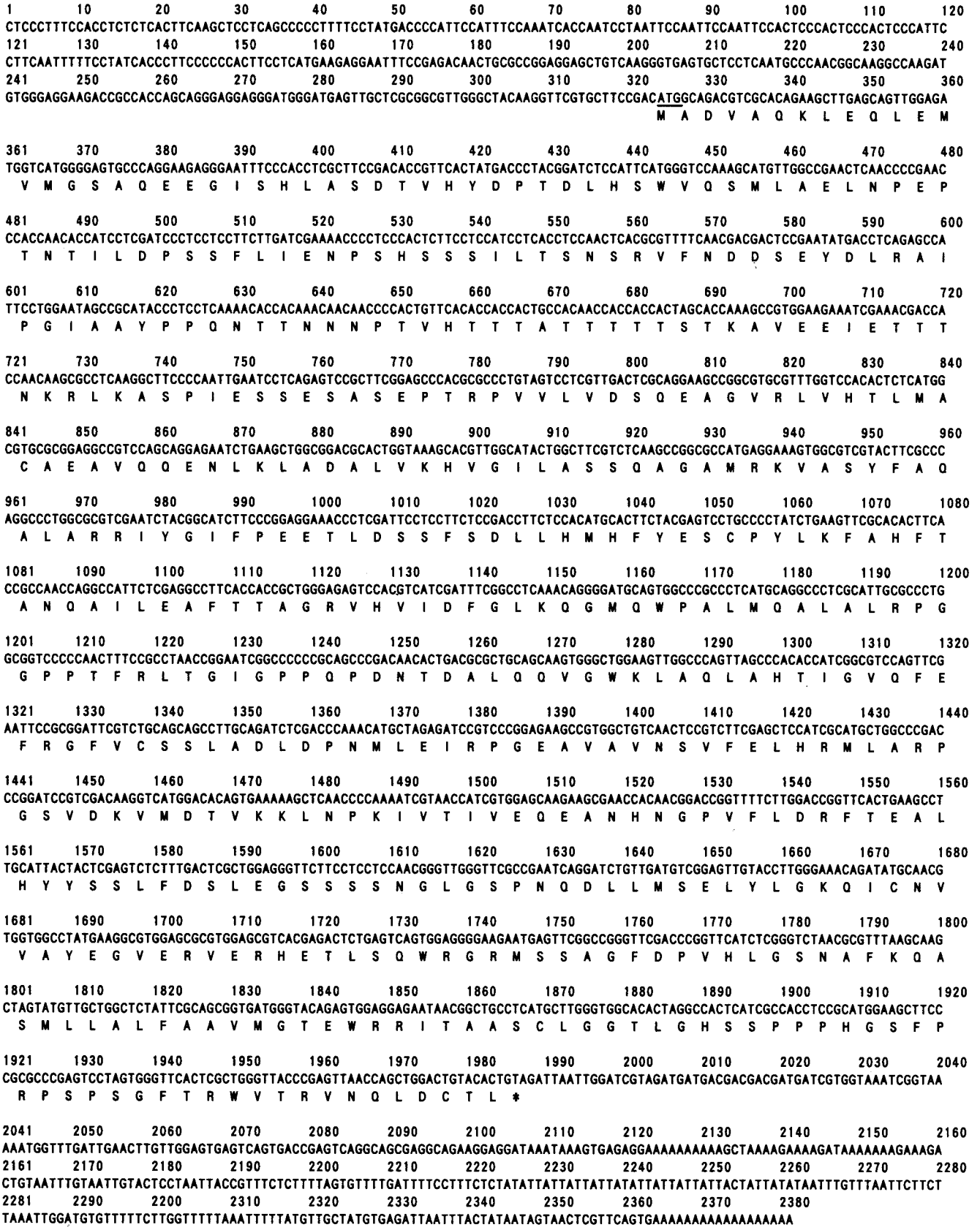


Fig. 20 The nucleotide sequence and deduced amino acid sequence of *GAUR1* cDNA. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

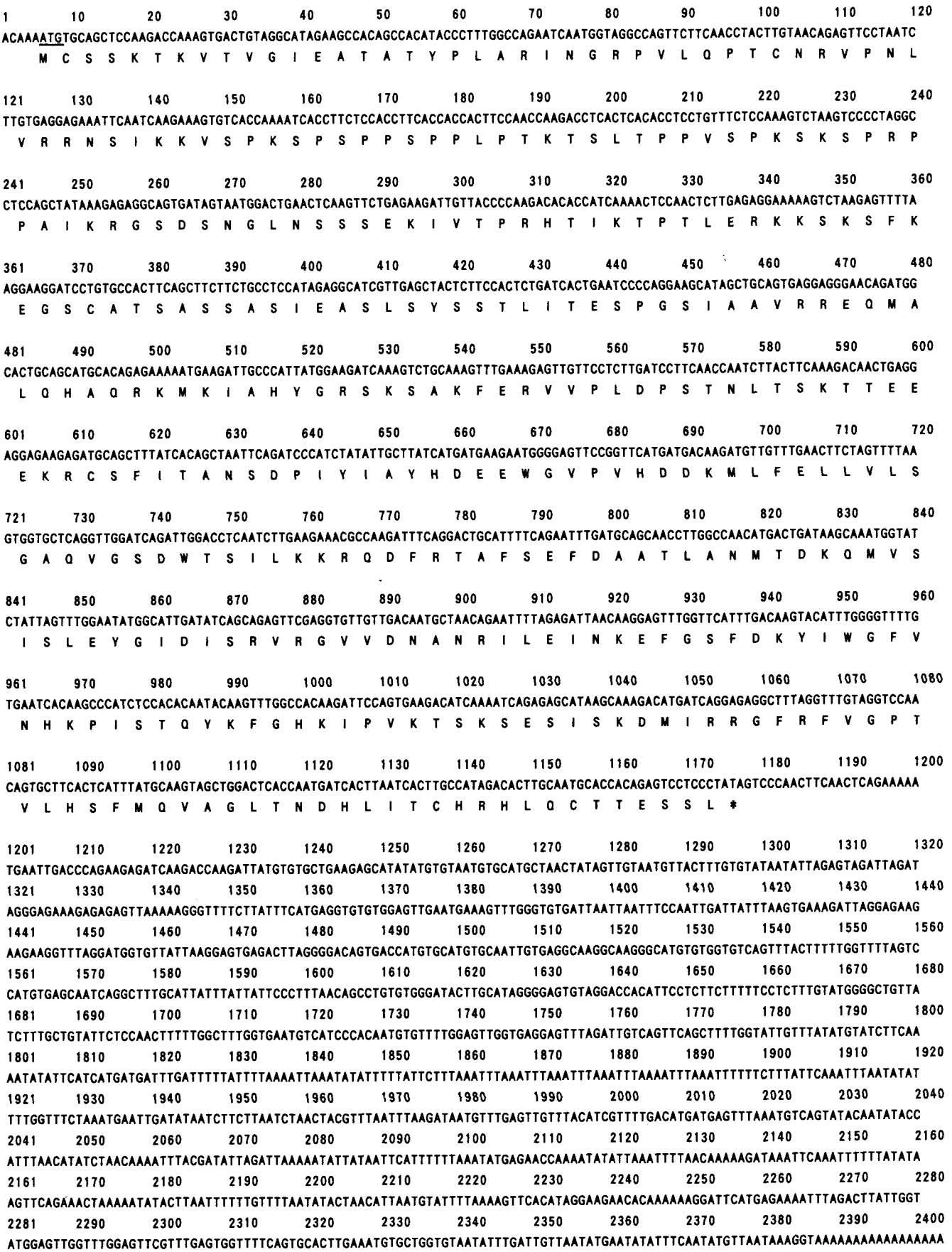


Fig. 21 The nucleotide sequence and deduced amino acid sequence of *GAUR2* cDNA. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

```
1          10         20         30         40         50         60         70         80
CTCTCTCTCTCTCTCTATCTCTCTCTCTCTCTCTATCTCTTAAGACACACACACACGCACTCACTCTTCACAACTGCG

81         90         100        110        120        130        140        150        160
ATTCACGCCAATGGATCGCAACAGTGTCTTCTCTCTCGCGTTCATCTGCATTGTAATAGCCGGCGTTGGAGGCCAGTCTC
           M  D  R  N  S  V  F  S  L  A  F  I  C  I  V  I  A  G  V  G  G  Q  S  P

161        170        180        190        200        210        220        230        240
CGGCGTCAGCGCCGTCGAGCACACCGGCAACTCCCGCCGCTTCCACTCCTGCGGCAGCCCCTATCACACCAAAATCACCT
  A  S  A  P  S  S  T  P  A  T  P  A  A  S  T  P  A  A  A  P  I  T  P  K  S  P

241        250        260        270        280        290        300        310        320
GCTCCCGTTGCTTCCCCCAAATCCTCTCCTCCCGCAGCATCACCTAAGGCCGCAACCCCCGCATCGACGCCGGCGGCATC
A  P  V  A  S  P  K  S  S  P  P  A  A  S  P  K  A  A  T  P  A  S  T  P  A  A  S

321        330        340        350        360        370        380        390        400
TCCTCCAACCGCTACAGCTCCTGCCCCTGCGACCAAGCCTCCGGCAGCGTCTCCTCCGGCTGCAACTCCAGTGAGCTCCC
 P  P  T  A  T  A  P  A  P  A  T  K  P  P  A  A  S  P  P  A  A  T  P  V  S  S  P

401        410        420        430        440        450        460        470        480
CACCGGCTCCAGTTCCAGTGAGCTCTCCCCCAGCTCCGGTTCCGGTGAGTTCTCCCCCTGCACCTGTGCCGACAGTTGCT
  P  A  P  V  P  V  S  S  P  P  A  P  V  P  V  S  S  P  P  A  P  V  P  T  V  A

481        490        500        510        520        530        540        550        560
CCCGTAGTGGCGCCCACCACCCCTGTGACTCCGGCTCCGGCTCCGGGAAAGCACAAGAAGACTAAGAAGCACAGTGCTCC
P  V  V  A  P  T  T  P  V  T  P  A  P  A  P  G  K  H  K  K  T  K  K  H  S  A  P

561        570        580        590        600        610        620        630        640
GGCACCCTCACCGTCGTTGCTTGGTCCCCCTGCTCCACCCACAGGTGCTCCTGGTCCCAGCGAAGATGCTACTTCCCCTG
 A  P  S  P  S  L  L  G  P  P  A  P  P  T  G  A  P  G  P  S  E  D  A  T  S  P  G

641        650        660        670        680        690        700        710        720
GACCTGCATCTTCTGCCAACGATGAGAGTGGAGCAGAGACCATCATGTGCTTGAAGAAGATTCTAGGAGGCTTAGCTTTG
  P  A  S  S  A  N  D  E  S  G  A  E  T  I  M  C  L  K  K  I  L  G  G  L  A  L

721        730        740        750        760        770        780        790        800
GGCTGGGCTACCCTTGTTTTGGTCTTCTAGACAGACTTTGTTACTGTTATTATATTCTTGTATTGTATCATTTATTTCCT
G  W  A  T  L  V  L  V  F  *

801        810        820        830        840        850        860        870        880
TTTTTTTAATTTTTCCTTTTCTTTCTCACCCTGTTTTGGGTACACATTACATTTATTTACATTGTTACTTGGGACTCTAT

891        900        910        920        930
TATTCGATTGATGTCGTTGTCATTAAAAAAAAAAAAAAAAAA
```

Fig. 22 The nucleotide sequence and deduced amino acid sequence of *GAUR3* cDNA. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

```
1          10         20         30         40         50         60         70         80
ATCAAGAGGGGTTTTCTGATCATAGTGTTGTGCAAAATGGCTATGGCTAAGTTCCTTGCAGCTATGATCTTGGCTCTCAT
                                    M  A  M  A  K  F  L  A  A  M  I  L  A  L  I

81         90         100        110        120        130        140        150        160
TGCCATTTCCATGCTTCAAACCGTGGTCATGGCTGCAAATGGGCATGGAGACCACCTGAATGATAATAAGAGCAAATATG
 A  I  S  M  L  Q  T  V  V  M  A  A  N  G  H  G  D  H  L  N  D  N  K  S  K  Y  G

161        170        180        190        200        210        220        230        240
GAAGTGGGAGTCTCAAGAGTTACCAATGCCCATCACAATGCTCGAGGAGATGCAACAAGACCCAATACCACAAGCCCTGC
  S  G  S  L  K  S  Y  Q  C  P  S  Q  C  S  R  R  C  N  K  T  Q  Y  H  K  P  C

241        250        260        270        280        290        300        310        320
ATGTTTTTCTGTCAGAAGTGCTGCAGGAAGTGCCTGTGTGTTCCTCCGGGTTATTATGGTAATAAGGCTGTGTGCCCTTG
M  F  F  C  Q  K  C  C  R  K  C  L  C  V  P  P  G  Y  Y  G  N  K  A  V  C  P  C

321        330        340        350        360        370        380        390        400
CTACAACAACTGGAAGACCAAGGAGGGAGGACCCAAGTGCCCTTGAGCTTCAACCTCAACATCAGATGTTGCTTTCTTAT
 Y  N  N  W  K  T  K  E  G  G  P  K  C  P  *

401        410        420        430        440        450        460        470        480
ATATTTATCACTTCCTTCATTGTTCCATTTAAGGCACTAGCTTACTCCCCTACTATTCACATGTCCTAACTAAATGATAT

481        490        500        510        520        530        540        550        560
ATAGCCCCTTGTGGTGGCACTATGATGTATGTTTTCTTATGAGTTTTGGGATGTCCTCCCTCTAAGGACCACATCCGTAG

561        570        580        590        600        610        620        630        640
TATCACTCCTTAGGACTTGCTTTCCTTTAATGTGAGTGGCTTACTTATATCAATACATCATCTGTGGAACTTATTTCTAG

641        650        660
CTTAAAAAAAAAAAAAAAAAA
```

Fig. 23 The nucleotide sequence and deduced amino acid sequence of *GAUR4* cDNA. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

```
1         10         20         30         40         50         60         70         80
CCCAACTGCACCTTCTTTCAGCTTCTCACAAAAATCTCTCAAGATGGCTGGCATAGCATTCGGACGCTTCGATGATTCTT
                                            M  A  G  I  A  F  G  R  F  D  D  S  F

81        90         100        110        120        130        140        150        160
TTAGTTTTGCCTCCATCAAGGCCTACATTGCTGAGTTCATCTCAACCCTTCTCTTTGTTTTTGCTGGTGTCGGTTCAGCC
  S  F  A  S  I  K  A  Y  I  A  E  F  I  S  T  L  L  F  V  F  A  G  V  G  S  A

161       170        180        190        200        210        220        230        240
ATAGCCTATGCTAAGTTGACATCAGATGCAGCTCTTGATCCTGCTGGGTTGGTAGCTGTTGCCATTGGCCATGGTTTTGC
I  A  Y  A  K  L  T  S  D  A  A  L  D  P  A  G  L  V  A  V  A  I  G  H  G  F  A

241       250        260        270        280        290        300        310        320
TCTCTTCGTTGCTGTTTCAGTCGGAGCCAACATTTCTGGTGGCCATGTTAACCCTGCTGTGACCTTTGGGTTGGCTCTTG
 L  F  V  A  V  S  V  G  A  N  I  S  G  G  H  V  N  P  A  V  T  F  G  L  A  L  G

321       330        340        350        360        370        380        390        400
GAGGTCACATCACCATCCTCACTGGTCTCTTCTACTGGATTGCGCAGCTCCTTGGCTCCATAGTGGCCTGCTTTCTCCTC
  G  H  I  T  I  L  T  G  L  F  Y  W  I  A  Q  L  L  G  S  I  V  A  C  F  L  L

401       410        420        430        440        450        460        470        480
AAGTTTGTCACTGGCTATAGTATTCCTATCCACGGTGTTGCTGCGGGAGTTGGAGCCGGAGAAGGAGTTGTTACTGAGAT
K  F  V  T  G  Y  S  I  P  I  H  G  V  A  A  G  V  G  A  G  E  G  V  V  T  E  I

481       490        500        510        520        530        540        550        560
CATCATCACATTTGGATTAGTGTACACAGTGTATGCTACAGCTGCAGACCCAAAGAAGGGTTCACTTGGTACAATTGCAC
 I  I  T  F  G  L  V  Y  T  V  Y  A  T  A  A  D  P  K  K  G  S  L  G  T  I  A  P

561       570        580        590        600        610        620        630        640
CCATTGCCATAGGTTTCATCGTTGGTGCAAACATCTTAGCTGCAGCACCATTTTCTGGGGGATCCATGAACCCTGCACGT
  I  A  I  G  F  I  V  G  A  N  I  L  A  A  A  P  F  S  G  G  S  M  N  P  A  R

641       650        660        670        680        690        700        710        720
TCCTTTGGCCCTGCAGTTGTCAGCGGTGACTTTCATGACAACTGGATCTACTGGATTGGCCCTCTTGTTGGTGGTGGTTT
S  F  G  P  A  V  V  S  G  D  F  H  D  N  W  I  Y  W  I  G  P  L  V  G  G  G  L

721       730        740        750        760        770        780        790        800
GGCTGGTCTTATCTACACCTATGCCTTCATTCCCACCCAGCACCATGCACCTCTCGCCACTGATTTTTGATTCACTCAAT
 A  G  L  I  Y  T  Y  A  F  I  P  T  Q  H  H  A  P  L  A  T  D  F  *

801       810        820        830        840        850        860        870        880
AATACCCTTTGTTAATCGCATCATGGTTTAGTTTCTATGTAATAAAGGAGGAAAACTCAGTCTTGTTTTTCCTTCTTTTC

881       890        900        910        920        930        940        950        960
ATCTCAGCCTTTTCTTTTTCATTTTGCTTTTAATGTAAAGTTGGAGTCATTGTTTTCTTGTACGAATTCATGAGGTTGCT

961       970        980        990        1000       1010       1020
GATGTAGTTTTTCTTAAAACATTGTTGTATCTCATTTATAGCTTAAAAAAAAAAAAAAAAAA
```

Fig. 24 The nucleotide sequence and deduced amino acid sequence of *GAUR5* cDNA. An underline indicates a start codon and an asterisk indicates a stop codon for the longest ORF as the putative coding region.

a.

```
SCR    378 PLVKFSHFTANQAIQEAFEKEDSVHIIDLDIMQGLQWPGLFHILASRPGGPPHVRLTGLG 437
GAUR1  245 PYLKFAHFTANQAILEAFTTAGRVHVIDFGLKQGMQWPALMQALALRPGGPPTFRLTGIG 304
```

b.

```
SCR    530 FLGRFVEAIHYYSALFDSLGAS 551
GAUR1  405 FLDRFTEALHYYSSLFDSLEGS 426
```

Fig. 25 Comparison of two homologous region of the deduced amino acid sequences of SCARECROW (SCR) of *Arabidopsis* and GAUR1 of azuki bean. Optimization of alignment was achieved with the ClustalW program. Identical amino acids of SCR and GAUR1 are shaded. An iunderline shows VHIID domain in SCR sequence.

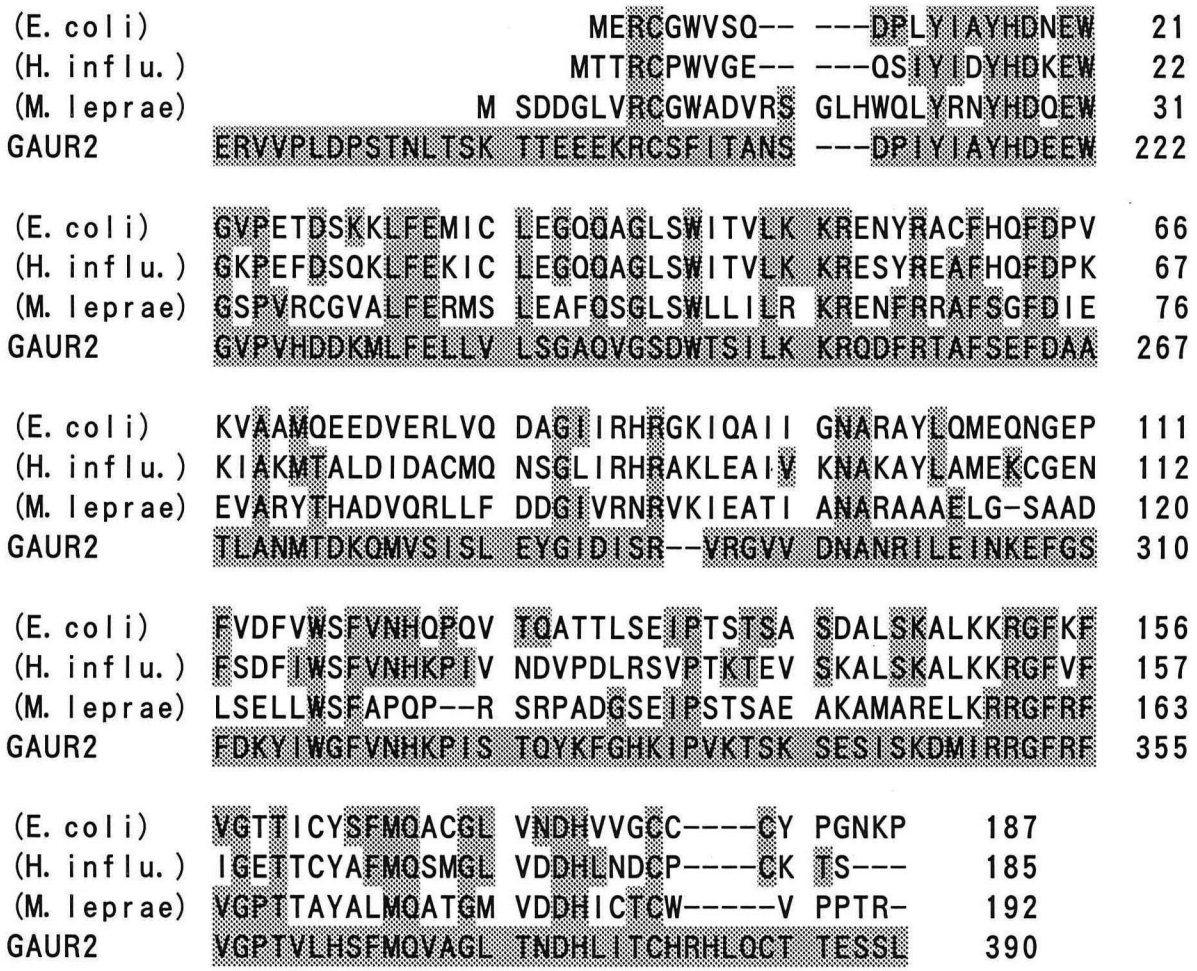


Fig. 26 Comparison of the amino acid sequences deduced from entire sequences of DNA- 3-methyladenineglycosidese genes in *E. coli*, *H. inflienzae* and *M. leprae* and partial sequence of GAUR2 in azuki bean. Optimization of alignment was achieved with the ClustalW program. Dashes indicate gaps introduced to maximize alignment. GAUR2 sequence and identical amino acids of DNA-3-methyladenineglycosidese in *E. coli*, *H. inflienzae* and *M. leprae* with those of GAUR2 are shaded.

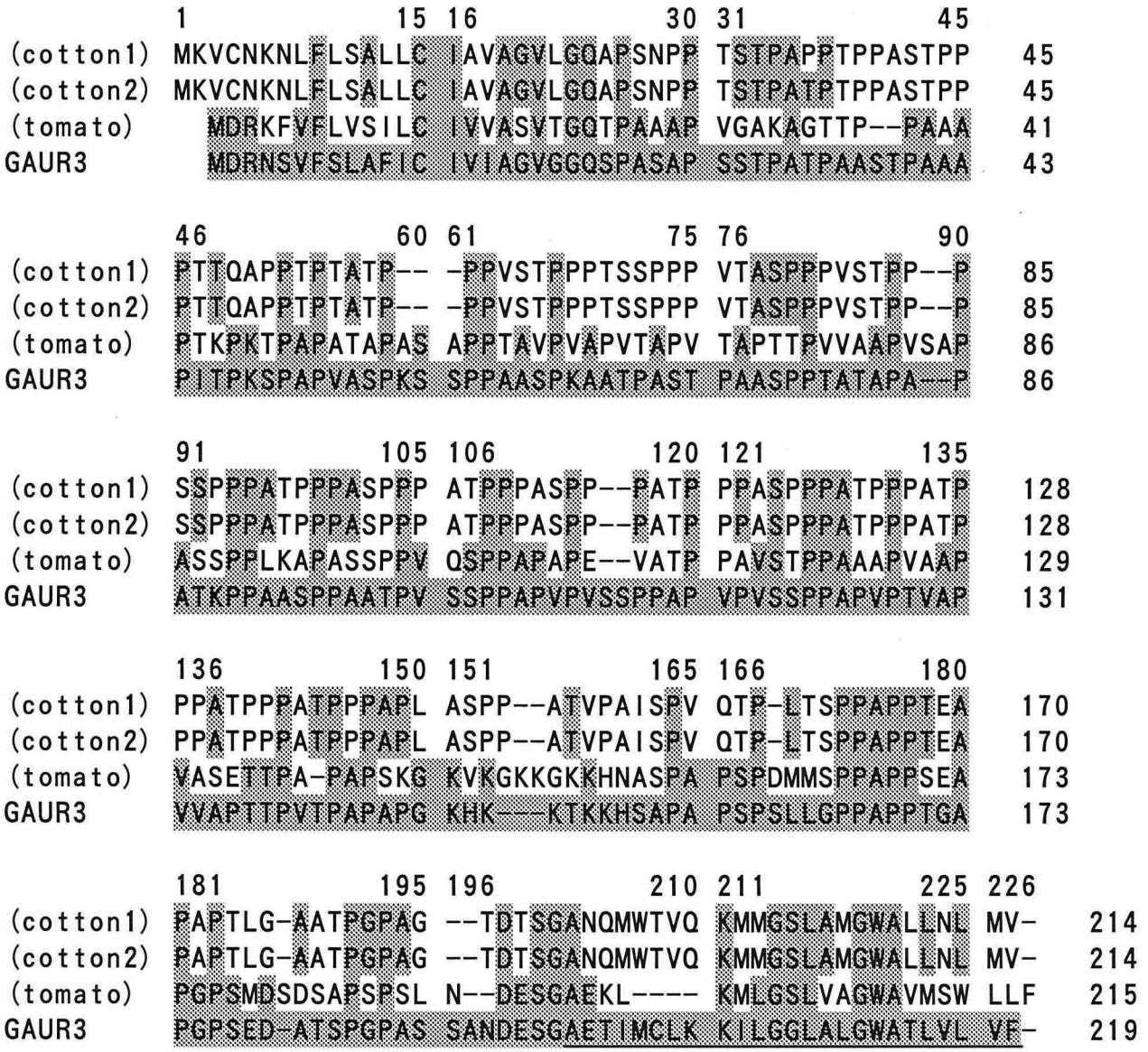


Fig. 27 Comparison of the deduced amino acid sequences of ploline-rich cell wall proteins in cotton fiber, probable arabinogalactan protein in tomat fruits and GAUR3 in azuki bean epicotyls. Optimization of alignment was achieved with the ClustalW program. Dashes indicate gaps introduced to maximize alignment. GAUR3 sequence and identical amino acids of cell wall proteins with those of GAUR3 are shaded. An underline shows a ptential transmembrane segment.

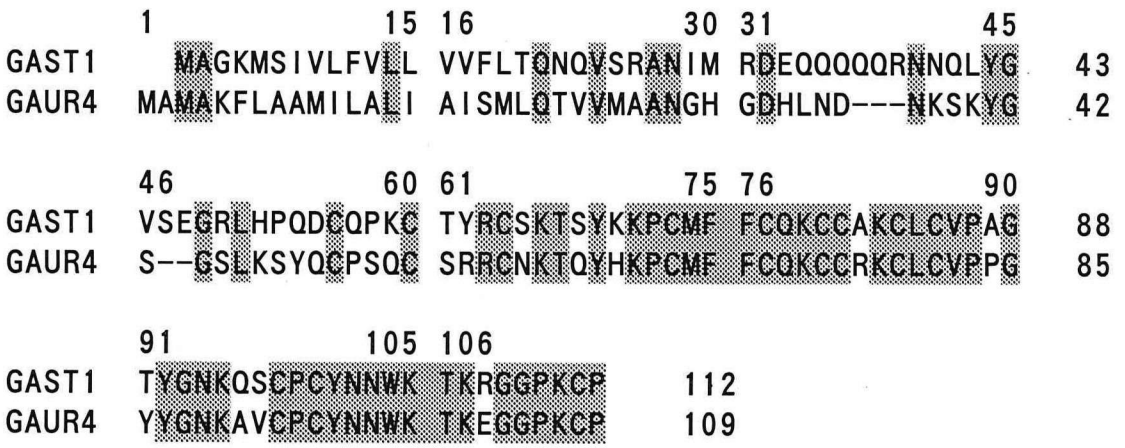


Fig. 28 Comparison of the deduced amino acid sequences of GAST1 in tomato shoot and GAUR4 in azuki bean epicotyls. Optimization of alignment was achieved with the ClustalW program. Dashes indicate gaps introduced to maximize alignment. Identical amino acids of GAST1 with GAUR3 are shaded.

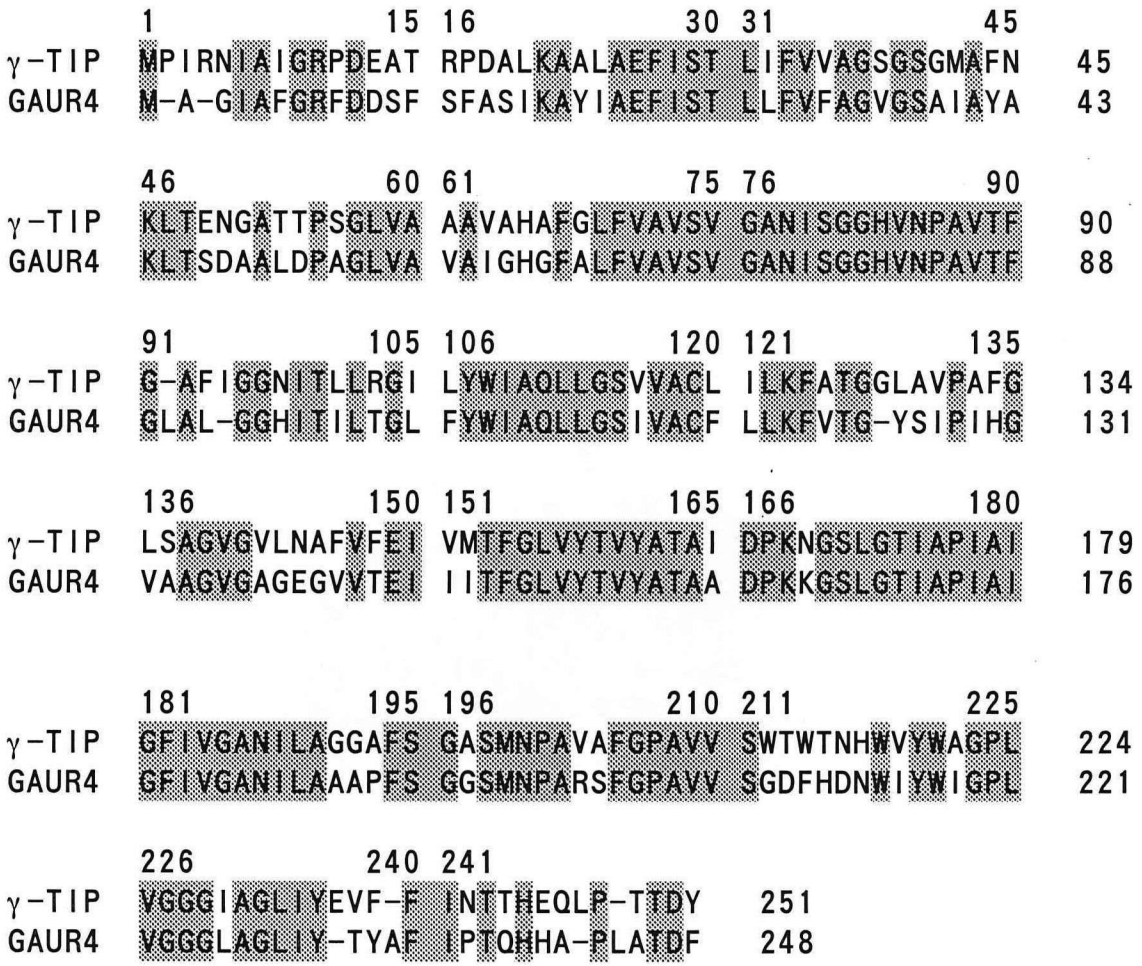


Fig. 29 Comparison of the deduced amino acid sequences of γ-TIP in *Arabidopsis* shoot and GAUR5 in azuki bean epicotyls. Optimization of alignment was achieved with the ClustalW program. Dashes indicate gaps introduced to maximize alignment. Identical amino acids of γ-TIP with GAUR5 are shaded.

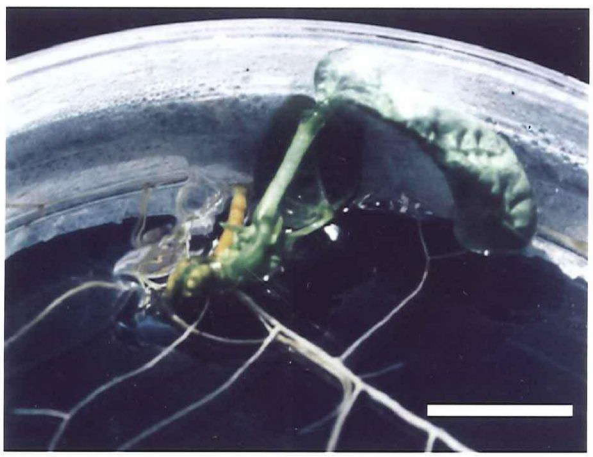


Fig. 30 Shoot regenerated from callus in azuki bean after 2-months incubation on shoot inducing medium. Bar=10 mm.