Gibberellin A3 Causes a Decrease in the Accumulation of mRNA for ACC Oxidase and in the Activity of the Enzyme in Azuki Bean (Vigna angularis) Epicotyls

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Differential screening, aimed at the isolation of cDNA clones of mRNAs whose accumulation is influenced by GA3, resulted in the isolation of a cDNA clone of an mRNA whose level was decreased by GA3 in segments of epicotyls of Vigna angularis. The putative protein encoded by this cDNA resembled the 1-aminocyclopropane-1-carboxylate oxidases (ACC oxidases) identified in other plant species (about 80% homology at the amino acid level). Thus, the corresponding gene was designated AB-ACO1 (azuki bean ACC oxidase). GA3 also decreased the activity of ACC oxidase in azuki bean epicotyls, but it did not decrease the rate of ethylene evolution. In fact, GA3 increased the rate of ethylene evolution and the level of ACC. Thus, GA3 seemed to increase the production of ethylene by promoting the synthesis of ACC.

Key words: ACC oxidase — Auxin — Ethylene — Differential screening — Gibberellin — Vigna angularis.

Higher plants have the ability to regulate their shape by regulating the way they develop in response to their environment. The control of the direction of cell expansion is an important feature of the environmental control of plant morphogenesis. Plant hormones, such as auxin, gibberellins and ethylene, regulate the shape of cells in the stems of higher plants. Auxins cause the enlargement of stem cells while gibberellins promote the longitudinal expansion and inhibit the lateral expansion of cells in the stems of various higher plants. By contrast, ethylene promotes the lateral expansion and inhibits the longitudinal expansion of cells (Shibaoka 1991). The shape of stem cells seems, thus, to be determined by the balance between the actions of these plant hormones. Therefore, clarification not only of the mechanisms of action of plant hormones at the molecular level but also of the effects of each plant hormone on the action and biosynthesis of the others is indispensable if we are to understand the regulation of plant morphogenesis.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AOA, amino-oxyacetic acid.

The nucleotide sequence reported in this paper has been submitted to DDBJ/EMBL/GenBank with accession number AB002667.

Azuki bean seedlings have proved to be very useful plant materials for studies of the effects of gibberellins on stem elongation. Gibberellins promote the elongation of stems of various plant species when they are applied to intact plants but they often fail to promote the elongation of excised stem segments. However, GA3 effectively promotes the elongation of excised stem segments of azuki bean seedlings. Although GA4 applied alone does not induce the elongation of segments of azuki bean epicotyls, GA3 applied together with IAA promotes the elongation that is induced by IAA (Shibaoka 1972). The simultaneous application of GA3 and IAA is not always necessary for the promotion by GA3 of IAA-induced elongation. GA3 applied before treatment with IAA also promotes IAA-induced elongation. Since actinomycin D, an inhibitor of transcription, strongly suppresses the effect of GA3 applied before treatment with IAA (Kaneta et al. 1993), the GA3-induced synthesis of mRNA seems to be involved in the GA3-induced promotion of the IAA-induced elongation of epicotyls. In this study, we used the technique of differential screening to isolate genes whose levels of expression were altered by exogenous GA3. We isolated a cDNA for an mRNA whose level was reduced by GA3, and we focused our attention on this cDNA because the sequence of this cDNA exhibited extensive similarity to the entire sequence of genes for ACC oxidase, which is known to catalyze the synthesis of ethylene from ACC (Spanu et al. 1991, Hamilton et al. 1991). We also examined the effects of GA3 on the synthesis of ethylene in segments of azuki bean epicotyls.

Materials and Methods

Plant material—Seeds of azuki bean (Vigna angularis ‘Takara-wase’) were sown in moistened vermiculite. Seedlings were grown under continuous light (4,000 lux) at 27°C for 5 d.

Treatments—Segments were prepared by cutting epicotyls at sites 5 mm and 15 mm below the node of the first leaves. Twenty-five 10-mm segments of epicotyls 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, flasks with segments were sealed with silicone rubber stoppers and incubated under continuous light at 25°C.

Construction of cDNA libraries from azuki bean epicotyls—Total RNA was extracted from epicotyl segments, which had been treated with basal medium that contained IAA or with basal medium that contained IAA and GA3 for 2 h, by the SDS-phenol
method using an Extract-A-Plant kit (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.). Poly(A)^+ RNAs were concentrated with Oligotex-dT30 <super> 150</super> (Nippon Roche Co., Ltd., Tokyo, Japan and Synthetic Rubber Co., Ltd., Tokyo, Japan) and cDNA libraries were constructed in the zZAPII vector by use of a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.). A library of 5 x 10^6 pfu was obtained from segments treated with IAA and a library of 3 x 10^6 pfu was obtained from segments treated with IAA plus GA_3.

**Differential screening**—We prepared [a-32P]dCTP-labeled cDNA probes from poly(A)^+ RNA that had been extracted from segments treated with IAA for 2 h and from poly(A)^+ RNA that had been extracted from segments treated with IAA plus GA_3 for 2 h. Probes were allowed to hybridize with duplicated copies of the cDNA libraries on nylon membranes (Hybond-N; Amersham). The hybridization was performed in 5 x SSPE (1 x SSPE = 10 mM NaH2PO4, pH 7.4, 150 mM NaCl, 1 mM EDTA) that contained 0.5% SDS, 50% formamid and 500 µg ml^-1 salmon sperm DNA at 46°C for 16 h. Then the membranes were washed once with 1 x SSC (=150 mM NaCl, 15 mM tri-sodium citrate) that contained 0.1% SDS at 68°C, and three times with 0.2 x SSC that contained 0.1% SDS at 68°C. Signals were then detected with a Bio-Imager (BAS-2000; Fujifilm, Tokyo, Japan). cDNA clones were selected from 5 x 10^8 plaques in the cDNA libraries by comparing the signals on each membrane. The dependence on GA_3 of the expression of the mRNA represented by each isolated clone was confirmed by Northern blotting analysis. In this way, we obtained cDNA clones for mRNAs whose levels were decreased or increased by treatment with GA_3.

We focused our attention on one of these clones, which we designated AB-ACOI.

**Screening of the AB-ACOI gene**—The AB-ACOI cDNA that had been isolated by differential screening was subcloned into the pBluescript SK(−) vector by an in vivo excision method, using a ZAP-cDNA Synthesis Kit (Stratagene). The insert was excised with EcoRI and XhoI and labeled with [a-32P]dCTP using the Megaprime Labeling System (Amersham) and it was used to screen a library based on RNA that had been extracted from segments treated with IAA. Hybridization and washing of membranes were performed under the same conditions as described above. Forty cross-hybridizing plaques were excised as pBluescript SK(−) plasmids, and nine of them, as well as the clone that had been produced from ACC were determined by Northern blotting analysis. In this way, we obtained cDNA clones for mRNAs whose levels were decreased or increased by treatment with GA_3.

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**Northern blotting analysis**—Ten µg of total RNA, extracted from segments treated with basal medium, with basal medium plus GA_3, with basal medium plus IAA and GA_3, were fractionated by electrophoresis on a 1% agarose gel that contained 0.66 M formaldehyde. Gels were stained with ethidium bromide and the stained gels were photographed. Then the separated RNAs were blotted onto nylon membrane (Hybond-N; Amersham). The synthesis of the probe, hybridization and washing of membranes were performed under the same conditions as described above for screening of the ABACOI gene. Signals were detected with the BAS-2000 system.

**Sequence analysis**—Sequencing of cDNAs that had been subcloned into the pBluescript SK(−) vector was performed with a Dye Primer Cycle Sequencing Kit, FS (Applied Biosystems, Inc., Foster City, CA, U.S.A.) and an automated DNA sequencer (model 373S; Applied Biosystems, Inc.).

**Quantitation of 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 with 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 a vacuum for 2 minutes for infiltration of ACC and AOA into the segments. One hour later, segments were sealed in 5-ml glass vials and incubated at 25°C. One ml of gas from each vial was sampled after a further hour and the amount of ethylene in the sampled gas was measured by gas chromatography.

**Quantitation of the evolution of ethylene**—One ml of gas was sampled at 2-h intervals from an Erlenmeyer flask in which epicotyl segments had been floated on basal medium or on basal medium that contained plant hormones. The amount of ethylene in each sample of gas was determined by gas chromatography.

**Results**

**Down-regulation of the expression of a gene by GA_3**—Differential screening allowed us to isolate several cDNA...
clones for mRNAs whose levels were altered by GA₃. We focused on a clone for an mRNA whose level was reduced by GA₃. Figure 1 shows the effects of GA₃, IAA, and GA₃ plus IAA on time course of changes in the amount of the mRNA that was recognized by the ³²P-labeled probe prepared from this cDNA, as revealed by Northern blotting analysis. Although the mRNA of about 1.2 kb was barely detectable in the epicotyls of intact plants (Fig. 1a, initial), it became apparent in excised epicotyl segments that had been floated on basal medium for 2 h and its level contin-

![Image]

Fig. 2 The nucleotide sequence and deduced amino acid sequence of the cDNA that corresponded to an mRNA whose accumulation was reduced by GA₃. Underlining indicates an initiation codon and an asterisk indicates a termination codon in the longest ORF, namely, the putative coding region.
ued to increase during a 10-h incubation (Fig. 1a, BM), suggesting that the amount of mRNA transcribed from this gene increased in response to the stimulus of wounding and/or submergence. IAA had no effect on the expression of this gene: the time course of changes in the amount of this mRNA in segments treated with IAA was almost the same with that in segments treated with basal medium only (Fig. 1a, BM and +IAA). GA3 decreased the wound- and/or submergence-induced increase in the accumulation of the mRNA but GA3 did not completely suppress the increase (Fig. 1a, Initial, +GA3). In the presence and in the absence of IAA, the amount of this mRNA was smaller in GA3-treated segments than in GA3-untreated segments (Fig. 1a, compare BM with +GA3 and +IAA with +IAA +GA3).

Sequence of the AB-ACOl cDNA—We isolated forty positive cDNA clones from 50,000 plaques of the unamplified cDNA library that had been constructed from mRNAs purified from segments that had been treated with IAA alone, using a probe derived from the cDNA of the GA3-down-regulated gene that we had identified by differential screening. The nucleotide sequences of nine of these clones and that of the original cDNA clone were determined. The three longest clones had an identical sequence of 1,184 bp (Fig. 2), in agreement with the results of the Northern blotting analysis that revealed an mRNA of about 1.2 kb.

The longest cDNA included the longest open reading frame (ORF, from position 45-47 of the ATG triplet to position 975-977 of the TAG triplet), which encoded 310 amino acids (Fig. 2). All ten cDNA clones that sequenced had a sequence identical to that shown in Figure 2, though their total lengths were different.

The cDNA exhibited extensive sequence similarity to genes for 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) that had been identified in various higher plant species (about 80% identity and more than 90% similarity). Thus, we designated this cDNA AB-ACOl (azuki bean ACC-oxidase). Figure 3 shows the alignment of amino acids deduced from nucleotide sequences of genes in the ACC oxidase family, such as Pch313 from peach fruits (Callahan et al. 1992), which was found to be the gene most similar to AB-ACOl (81% identity, 92% similarity); pGEFE (83% identity, 94% similarity) from geranium leaves (Wang et al. 1994); pTOM5 (82% identity, 92% similarity) and pTOM13 (79% identity, 92% similarity) from tomato fruits (Holdsworth et al. 1987); and AB-ACOl, isolated from azuki bean epicotyls in this study.

Effect of GA3 on ACC oxidase activity—Since the product of the AB-ACOl gene, whose expression was suppressed by GA3, was postulated to be ACC oxidase in azuki bean plants, we examined whether or not GA3 caused a decrease in the activity of ACC oxidase in segments of azuki bean epicotyls. Experiments were performed both in the presence and in the absence of IAA (Table 1). As shown in Table 1, the activity of ACC oxidase increased during incubation with basal medium, doubling during a 6-h incubation and tripling during an 8-h incubation. IAA had no obvious effect on the wound- and/or submergence-induced increase in the activity of ACC oxidase. The difference in the activity of ACC oxidase between segments treat-
GA3 inhibits expression of gene for ACC oxidase

Table 1 Inhibition by GA3 of increases in the activity of ACC oxidase

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Basal medium</th>
<th>+GA3</th>
<th>+IAA</th>
<th>+IAA+GA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>74.7± 5.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>160.9±19.5</td>
<td>128.9±1.4</td>
<td>125.0±10.8</td>
<td>91.1±3.2</td>
</tr>
<tr>
<td></td>
<td>128.9±1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 h</td>
<td>236.4±7.1</td>
<td>187.1±7.0</td>
<td>237.2±21.1</td>
<td>101.0±15.8</td>
</tr>
<tr>
<td></td>
<td>236.4±7.1</td>
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</table>

Increase in the activity

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Basal medium</th>
<th>+GA3</th>
<th>+IAA</th>
<th>+IAA+GA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>86.2</td>
<td>54.2</td>
<td>50.3</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td></td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>8 h</td>
<td>161.7</td>
<td>112.4</td>
<td>162.5</td>
<td>26.3</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td></td>
<td></td>
<td>82</td>
</tr>
</tbody>
</table>

Inhibition of the increase in the activity (%)

Ethylene produced, nl h⁻¹ (25 segments)⁻¹. Averages of results for three sets of 25 segments each are shown with standard errors.

ed with basal medium and those treated with basal medium that contained IAA after a 6-h incubation was not significant at the 10% level (Table 1). By contrast, GA3 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 GA3 was not significant at the 10% level after a 6-h incubation, the difference observed after an 8-h incubation was significant at the 0.8% level (Table 1). IAA alone had almost no effect on the activity of ACC oxidase but it greatly enhanced the effect of GA3. Together, IAA and GA3 inhibited the increase in the activity by about 80%, whereas GA3 alone inhibited the increase by about 30% to 40% (Table 1).

Effects of GA3 on ethylene evolution and the accumulation of ACC—To explore the possibility that the GA3-induced suppression of the increase in the activity of ACC oxidase might cause a decrease in ethylene evolution, we examined the effect of GA3 on the evolution of ethylene in segments of azuki bean epicotyls in the presence and in the absence of IAA (Fig. 4). The rate of ethylene evolution in freshly prepared segments was low and the rate did not increase in segments that were treated with basal medium or with basal medium that contained GA3 alone (Fig. 4). However, the rate clearly increased in segments treated with basal medium that contained IAA (Fig. 4). The increase became evident during the first 2 h of treatment and reached a maximum value between 6 and 8 h after the start of treatment.
GA \textsubscript{3} inhibits expression of gene for ACC oxidase

amount (Fig. 4). GA\textsubscript{3} applied together with IAA enhanced the IAA-induced increase in the rate of ethylene evolution and the enhancement was marked from 6 to 8 h and from 8 to 10 h after the start of treatment (Fig. 4), in spite of the result that GA\textsubscript{3} and IAA synergistically inhibited the wound- and/or submergence-induced increase in the activity of ACC oxidase (Table 1). In an attempt to explain why GA\textsubscript{3} applied together with IAA increased the rate of ethylene evolution, even though it caused a decrease in the activity of ACC oxidase, we measured the amount of ACC in epicotyl segments. The amount of ACC in freshly prepared segments was low, and treatment with basal medium or basal medium that contained GA\textsubscript{3} scarcely caused any accumulation of ACC (Fig. 5). IAA alone increased the level of ACC in the epicotyl segments, just as it increased the rate of ethylene evolution, and the amount of ACC reached a maximum value after 8 h of treatment (Fig. 5). GA\textsubscript{3} applied together with IAA enhanced the accumulation of ACC caused by IAA (Fig. 5). The enhancement became clear from 4 to 6 h after the start of treatment (Fig. 5), when the enhancement by GA\textsubscript{3} of the production of ethylene was not yet apparent (Fig. 4).

Discussion

A gene for ACC oxidase, pTOM13, was isolated initially as a ripening-related gene from tomato fruits (Holdsworth et al. 1987). Antisense suppression of pTOM13 in transgenic tomato plants resulted in a decrease in the rate of ethylene evolution and in the ACC oxidase activity of leaf disks that had been excised from the plants (Hamilton et al. 1990). Furthermore, the function of this gene was confirmed directly when the product of pTOM13, expressed in yeast and in oocytes of Xenopus, was shown to catalyze the synthesis of ethylene from ACC (Spanu et al. 1991, Hamilton et al. 1991). Since the \textit{AB-ACO1} gene exhibits extensive homology to the pTOM13 gene and to genes for ACC oxidase from other plant species (Fig. 3), we deduced that the \textit{AB-ACO1} gene encodes ACC oxidase in azuki bean epicotyls.

GA\textsubscript{3} suppressed increases in the accumulation of \textit{AB-ACO1} mRNA and in the activity of ACC oxidase in segments of azuki bean epicotyls (Fig. 1 and Table 1). However, the decrease in the accumulation of the mRNA and in the activity of ACC oxidase in the segments of azuki bean epicotyls did not cause a decrease in ethylene evolution (Fig. 4). The decrease in the accumulation of \textit{AB-ACO1} mRNA and in the activity of ACC oxidase did not seem to be responsible for the GA\textsubscript{3}-induced promotion of epicotyl elongation since GA\textsubscript{3} applied together with IAA increased the rate of ethylene evolution (Fig. 4), as well as the amount of ACC in the epicotyl segments (Fig. 5). Thus, GA\textsubscript{3} seemed to increase the rate of ethylene production via promotion of the synthesis of ACC, in spite of decreases in the amount of \textit{AB-ACO1} mRNA and in the activity of ACC oxidase. GA\textsubscript{3} inhibited the wound- and/or submergence-induced increase in the amount of \textit{AB-ACO1} mRNA and in the activity of ACC oxidase (Fig. 1 and Table 1). However, GA\textsubscript{3} did not inhibit the evolution of ethylene. GA\textsubscript{3} applied together with IAA, actually promoted the IAA-induced increase in the rate of ethylene evolution and it also promoted the increase in the amount of ACC (Fig. 4, 5). These results suggest that ACC oxidase is not the rate-limiting enzyme in the synthesis of ethylene in segments of azuki bean epicotyls. It has been reported that IAA induces the synthesis of ACC synthase and an increase in the activity of the enzyme in hypocotyls of mung bean (Yoshii and Imaseki 1981) and of winter squash (Nakagawa et al. 1991). It seems likely that IAA induces the synthesis of ACC synthase and that GA\textsubscript{3} promotes the IAA-enhanced induction of the synthesis of ACC synthase in segments of azuki bean epicotyls.

Differential screening using excised segments of azuki bean epicotyls allowed us to isolate a cDNA clone of \textit{AB-ACO1}, a gene for a putative ACC oxidase in azuki bean plants, that is down-regulated by GA\textsubscript{3}. We also obtained several cDNA clones for other genes that are up-regulated or down-regulated by GA\textsubscript{3}. The characterization of such genes is now in progress.

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References


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