Gibberellin Induces α-Amylase Gene in Seed Coat of Ipomoea nil Immature Seeds

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Two full-length cDNAs encoding gibberellin 3-oxidases, InGA3ox1 and InGA3ox2, were cloned from developing seeds of morning glory (Ipomoea nil (Pharbitis nil) Choisy cv. Violet) with degenerate-PCR and RACEs. The RNA-blot analysis for these clones revealed that the InGA3ox2 gene was organ-specifically expressed in the developing seeds at 6–18 days after anthesis. In situ hybridization showed the signals of InGA3ox2 mRNA in the seed coat, suggesting that active gibberellins (GAs) were synthesized in the tissue, although no active GA was detected there by immunohistochemistry. In situ hybridization analysis for InAmy1 (former PnAmy1) mRNA showed that InAmy1 was also synthesized in the seed coat. Both InGA3ox2 and InAmy1 genes were expressed spatially overlapped without a clear time lag, suggesting that both active GAs and InAmy1 were synthesized almost simultaneously in seed coat and secreted to the integument. These observations support the idea that GAs play an important role in seed development by inducing α-amylase.

Key words: α-amylase; developing seed; in situ hybridization; Ipomoea nil (Pharbitis nil); gibberellin(s)

Phytohormone gibberellins (GAs) relate to many aspects of plant growth. Especially at the seed germination of cereals, the mechanisms of the perception and signal transduction of GAs have been extensively studied.1–5) Also in seeds of dicotyledonous plants, much information on the roles of GAs in seed germination has been accumulated gradually.6,7) On the other hand, many things remain unrevealed for the physiological meaning of GAs during a maturation of dicotyledonous seeds, in spite of the existence of a high amount of GAs there.

In our previous report,8) we proposed the active form of GAs in developing seeds of morning glory (Ipomoea nil*) might be involved in a degradation of starch granules via an induction of α-amylase genes as shown in the germination of cereals. This proposed role of GAs was based on studies describing that GAs were localized around starch granules of integument in the seeds, and the GA-inducible α-amylase was also localized at the same site as GAs did prior to the degradation of starch granules, assessed by an immunohistochemical analysis.

Surely, the immunohistochemistry brings us much useful information of localized target molecule itself. However the physiological importance of those immunohistochemical data depends on various factors; for example, the specificity of antibody, the detection limit of staining, the possibility of drift of the target molecules during fixation, and the fixation efficiency to neighboring tissues. It leads to that there are potentials that the immunohistochemical staining could not detect very low level of GAs and/or GA-inducible α-amylase, and that we have not detected the original sites of them because of their unpreventable drifts. Therefore, we planned this study to examine the site where the active form of GAs were synthesized and then to evaluate the importance of GAs in developing seeds, by using the in situ hybridization technique.

The active form of GAs are usually synthesized in plants by the action of GA 3-oxidase (GA3ox), categorized to a subfamily of 2-oxoglutarate-dependent dioxygenases. The GA3ox gene was firstly cloned with an Arabidopsis GA-deficient mutant by a heterologous expression technique,9,10) and the Arabidopsis genome contains at least 4 genes encoding GA 3-oxidase.11) Now besides clones from Arabidopsis, sequential information for this enzyme have been accumulated from several other plants; lettuce,7) pea,12) pumpkin,13) rice,14) spinach,15) tobacco,16) watermelon,17) and so on. However no GA 3-oxidase gene had been isolated from morning glory.

So in this report, we describe the isolation of full-
length cDNAs encoding GA 3-oxidase from developing seeds of morning glory and the investigation of expression patterns of the genes and that of InAmyl by in situ hybridization, and then we discuss the results compared with the information on an induction of α-amylase in cereal seeds.

Materials and Methods

Plant materials. Twenty to 25 morning glory (Ipomoea nil (Pharbitis nil) Choisy cv. Violet) seeds were imbibed for about 20 h, sown in a plastic planter (50 cm × 20 cm × 20 cm deep), and grown in a chamber at 25 to 27°C with alternating continuous light (13 h) and continuous dark (11 h). The developing fruits were harvested about 2 months after sowing, and used for the analyses. Each developing seed used for RNA-blot analysis or in situ hybridization was prepared by removing husks including carpel and placenta from fruits, and they were immediately frozen in liquid nitrogen.

Cloning of full-length cDNAs of GA 3-oxidase genes. Total RNA was extracted from the mixture of seeds at 4 to 24 DAA and cDNA was synthesized according to the method described previously. Based on the amino acid sequence of known homologous genes from other dicotyledonous plants, the following degenerate primer sets were designed; forward primer, 5'-AARMT-VATGGGYGMYGARGWTTYAC-3'; reverse primer, 5'-GARTCYGTRGNRSRCNAGCCAT-3'. The cDNA from developing seeds was used as template, and PCR was performed with thermal cycler (Takara, Kyoto); initiated condition: 94°C for 3 min; cycled condition: 94°C for 30 sec, 50°C for 1 min, and 72°C for 2 min (30 to 36 cycles). The reaction was ended after a 5-min incubation at 72°C. The PCR products were purified by 1.5% (w/v) agarose gel electrophoresis and submitted to a sequence analysis with a DNA sequencer (SQ5000E, Hitachi, Tokyo). Full-length cDNAs were obtained by 5'- and 3'-RACEs according to the previous method.

RNA-blot analysis. Total RNA was extracted from various tissues in addition to developing seeds according to the previous method. Total RNA (2-10 µg) was electrophoresed with 1% (w/v) agarose gel containing 5% (v/v) formaldehyde and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences). RNA probe corresponding to full-length cDNA of each gene was prepared with a DIG RNA labeling kit (Roche) and 1/1,000 to 1/2,000-diluted in the hybridization buffer shown in the manufacturer’s protocol. The procedures for hybridization and detection are the same as the previous ones.

Expression of recombinant InGA3ox2 in E. coli. The cDNA for the recombinant InGA3ox2 to be inserted into the pGEX-4T-2 vector (Amersham Biosciences) were prepared using the following PCR primers; forward primer: 5’-GTGAATCCATGTTCAACAAGGGACC-3’; reverse primer: 5’-GGGATCCACTTTTATTGCCTCCACTGC-3’. After being purified, the cDNA was inserted into the BamHI site of the pGEX-4T-2 vector. The pGEX plasmid harboring InGA3ox2 ORF-region was used to transform E. coli BL21 (DE3) pLysS-competent cells (Stratagene, CA, USA) according to the manufacturer’s protocol, before selecting the colonies on an Luria Bertani (LB) plate containing 1.5% (w/v) agar and 100 µg ml⁻¹ ampicillin. A single colony was selected and grown overnight at 26°C. A 2.0 ml- aliquot of the culture was inoculated into 100 ml of fresh LB broth containing 100 µg ml⁻¹ ampicillin and 2% (w/v) glucose, and shaken at 26°C. When cell density reached O.D. 0.6 (600 nm), the fusion protein was induced by removing glucose by centrifugation (1,000 g, 2 min) followed by addition of isopropyl-β-D-thiogalactoside to be 0.3 mm. After 5 h of incubation, the cells were collected by centrifugation (10,000 g, 10 min), and resuspended in 10 ml of DW containing 1 mg ml⁻¹ lysozyme. After 10 min of incubation, the suspension was frozen and kept at −80°C overnight, thawed in water (30°C), chilled on ice, and sonicated well. Soluble proteins were collected by centrifugation and stored at −80°C.

Enzyme assay. The reaction mixture was prepared by adding ca. 5 µg of crude soluble protein into 50 µl of 100 mM Tris-HCl (pH 7.6) containing 4.0 mM 2-oxoglutaric acid, 0.5 mM ferrous sulfate, 4.0 mM ascorbic acid, and 1 µg of GA₉, a substrate of GA 3-oxidase. After 1 h of incubation at 30°C, the enzymatic reaction was ended by adding 10 µl of 1 M acetic acid. The solution was applied to a Sep-Pak™ cartridge C18 column (1 ml, Waters, MA, USA), and the column was eluted with 3 ml of DW and 2.5 ml of methanol, successively. The methanol eluate was dried up in vacuo, and the concentrate was methylated with CH₃N₂ followed by trimethylsilylation in fresh N-methyl-Ntrimethylsilyl trifluoroacetamide (MSTFA). A portion of the reaction mixture was put through full-scan GC/MS (GC: HP-5890 ser.II; MS: Hitachi M-4100), equipped with a capillary column DB-1 (15 m × 0.25 mm i.d., 0.25 µm thick; J&W Scientific, CA, USA) under the following conditions; temperature program: 60°C (2 min), 60 to 300°C linear gradient at 15°C min⁻¹. He flow: 1 ml min⁻¹, ionization: EI (70 eV). Product (GA₁) was identified by comparing its MS and Kovats’ retention indices to an authentic one.

In situ hybridization. Approximately one-fifth of each developing seed of morning glory was cut off with a razor to speed the following fixation, and then the remains were fixed in a FAA solution [5% (v/v) formaldehyde, 50% (v/v) ethanol, and 0.9 M acetic acid] at 4°C overnight, dehydrated with ethanol and xylene,
Fig. 1. Alignment of Deduced Amino Acid Sequences of InGA3ox1 and InGA3ox2 with Those of Various Other Species.

The sequence information of InGA3ox1 and InGA3ox2 will be found in EMBL/DDBJ/GenBank on-line database services with the accession numbers AB099486 and AB099487, respectively. The deduced amino acid sequences of InGA3ox1 and InGA3ox2 were aligned by a program of Genetix (ver. 9.0) with those of GA 3-oxidase from some other plants: AtGA3ox1 (At1g15550 as the AGI codes from Arabidopsis Genome Initiative http://www.arabidopsis.org/agi.html) from *A. thaliana*, ClGA3ox (accession no. AF074710) from watermelon (*Citrullus lanatus*), CmGA3ox (AJ302040) from pumpkin (*Cucurbita maxima*), LeGA3ox1 (AB010991) from tomato (*Lycopersicon esculentum*), LsGA3ox1 (AB012205) from lettuce (*Lactuca sativa*), NsGA3ox1 (AF494089) from *Nicotiana sylvestris*, NtGA3ox (AB032198) from *N. tabacum*, OsGA3ox1 (AB054084) from rice (*Oryza sativa*), PsGA3ox (AF001219) from Pea (*Pisum sativum*), SoGA3ox1 (AF506280) from spinach (*Spinacia oleracea*), some of which were renamed from the original gene name as a matter of convenience. A signature of deduced 2-oxoglutarate-binding site was marked with an open square and a signature of three deduced ferrous ion-binding residues were gray-lined. Dots or asterisks indicate similar or identical amino acid, respectively.

The alignment of these sequences is shown in Fig. 1. The deduced amino acid sequences of InGA3ox1 and InGA3ox2 were aligned with those of GA 3-oxidase from various other plants.

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Genetics of GA3ox1 and GA3ox2 in various plants.
was done at 50°C according to the manufacturer’s protocol. Hybridization labeling kit (Roche) and T7 and SP6 RNA polymerase according to the manufacturer’s protocol. Hybridization was done at 50°C overnight in a solution consisting of 55% (v/v) formamide, and 45% (v/v) 1 mM Tris-HCl (pH 8.0) containing 0.3 M sodium chloride, 0.1 mM EDTA, 1 x denhard’s, 10% (w/v) dextran sulfate, and 1 to 5 µg RNA probe ml⁻¹. The hybridized samples were treated with 20 µg ml⁻¹ RNase A solution at 37°C for 30 min and washed twice in SSC solution at 50°C for 20 min, and hybridized probes were made visible by the same antisera and substrate used for the RNA-blot analyses described above.

Results and Discussion

Cloning of GA 3-oxidase cDNAs from developing seeds of morning glory

Using the degenerated primers designed from the well-conserved sequences in known GA 3-oxidase genes, PCR fragments were amplified from cDNAs prepared from the mixture of the seeds at different developmental stages, at 4 to 24 DAA. We successfully cloned two full-length cDNAs, designated InGA3ox1 and InGA3ox2, by 3'- and 5'-RACEs. InGA3ox1 cDNA (1.2 kb, accession no. AB099486) encoded a polypeptide of 372 amino acid residues, and InGA3ox2 cDNA (1.3 kb, accession no. AB099487) encoded a polypeptide of 368 amino acid residues in their ORF regions. The identity in terms of deduced amino acid sequence between InGA3ox1 and InGA3ox2 was ca. 44%. Figure 1 shows the alignment analysis of InGA3ox1 and InGA3ox2 in comparison with other GA 3-oxidases. The deduced amino acid sequences of InGA3ox1 and InGA3ox2 had a signature of a deduced 2-oxoglutarate-binding site (open square) and that of ferrous ion-binding sites (gray lines), showing that InGA3ox1 and InGA3ox2 are categorized as 2-oxoglutarate-dependent dioxygenase. A genomic DNA blot analysis showed two or three major bands in the digests of the genome DNA with EcoRI, HindIII, or KpnI, indicating more than two homologous genes exist in the genome of I. nil.

RNA-blot analyses of InGA3ox1 and 2

Referring to the sequences of InGA3ox1 and 2, RNA riboprobes specific for each GA 3-oxidase gene were prepared. The temporal expression pattern of InGA3ox2 is examined using the seeds at different developmental stages from 6 to 18 DAA, and shown in Fig. 2(a). A slight signal of InGA3ox2 mRNA was already recognized at 6 DAA, and it clearly increased by 18 DAA. Figure 2(b) shows the RNA-blot analysis of InGA3ox2 in the different organs. The signal of InGA3ox2 was detected only in developing seeds, but not in leaf, root, stem, or flower, while no signal of InGA3ox1 was detected in any organ when similarly examined (data not shown). Other homologous genes should exist for the stem elongation because no mRNA of InGA3ox1 and 2 was detected in stem.

Functional assay of recombinant InGA3ox2-GST

The recombinant InGA3ox2 protein with a GST tag was prepared by using an E. coli expression system. The SDS-PAGE of the crude extract containing InGA3ox2-GST fusion protein showed a major band at 66 kDa that corresponded to the deduced molecular mass of the fusion protein, and this band was missing in the control crude extract containing GST protein (Fig. 3). The crude extract containing GST protein (Fig. 3). The crude extract containing GST protein (Fig. 3). The crude extract containing GST protein (Fig. 3).
extracts obtained by centrifugation of cell lysates were examined by an enzyme assay without further purification, using GA$_9$ as a substrate. We identified GA$_4$, an expected metabolite, in an extract from the assay mixture with InGA3ox2-GST as its methyl ester-trimethylsilyl ether, because its mass spectrum and Kovats’ retention index were in good agreement with the authentic’s: m/z 418 [M$^+$] (18%), 386 (29%), 328 (37%), 289 (50%), 284 (100%), 261 (27%), 225 (78%), 201 (33%), and 129 (20%). No trace of the GA$_4$ derivative was detected in the extract from the assay mixture with the control extract containing GST protein.

This clearly confirms that InGA3ox2 encodes a GA 3-oxidase which is expressed in the developing seeds with high organ specificity.

**Spatial localization of InGA3ox2 mRNA and InAmy1 mRNA during seed development**

The localization of InGA3ox2 mRNA in a developing seed of morning glory was made visible by *in situ* hybridization with the specific probe. As shown in Figs. 4(a)–(h), specific staining due to InGA3ox2 mRNA was observed in a part of the seed coat at 9–15 DAA (Figs. 4(b)–(d)), whereas there was no staining in the control

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**Fig. 4.** Temporal Expression Analyses of InGA3ox2 and GA-Inducible $\alpha$-Amylase Gene InAmy1 in the Developing Seed at 6 to 15 DAA by *in Situ* Hybridization.

The top row shows staining with the antisense probe for InGA3ox2, and the second row from the top shows each a control staining with the sense probe instead of the antisense one. The third row from the top shows staining with the antisense probe for InAmy1, and the bottom row shows each a control staining with the sense probe. Arrows indicate each specific staining due to the expression of InGA3ox2 mRNA or InAmy1 mRNA, respectively. (a)(e)(i)(m), 6 DAA; (b)(f)(j)(n), 9 DAA; (c)(g)(k)(o), 12 DAA; (d)(h)(l)(p), 15 DAA; (SC), seed coat; (IT), integument. Bar is 10 $\mu$m.
experiments with a sense probe for InGA3ox2 (Figs. 4(f)–(h)). This specific staining gradually expanded to the whole area within the seed coat by 15 DAA, but did not permeate to any other part of the seed. 

**In situ** hybridization for InAmy1 mRNA showed antisense-specific staining in a part of the seed coat that was not observed in the control staining with a sense probe. The spatial expression pattern of InAmy1 (Figs. 4(i)–(p)) entirely overlapped with that of InGA3ox2, supporting a close connection between active GAs and InAmy1 in the development of seeds.

**Role of GA in a developing seed of morning glory**

We have reported that physiologically active GAs, GA\(_1\) and GA\(_3\), localized in integument of developing seeds of *I. nil*, spatially overlapping with the \(\alpha\)-amylase (InAmy1) that was inducible by GA. Based on the observation that appearance of these GAs preceded to that of InAmy1, and the adequate time lag between the appearance of InAmy1 and degradation of starch granules in the integument, we proposed that a similar process that occurs in \(\alpha\)-amylase induction by GA and starch degradation in endosperm at the germination of cereal grain, proceeds in developing seeds of morning glory. In this paper, we show by *in situ* hybridization that syntheses of active GAs and \(\alpha\)-amylase (InAmy1) occurred in seed coat where neither GA\(_{1/3}\) nor InAmy1 was detected by immunohistochemistry. Although there still remains the possibility of the drift of the molecules during the fixation for immunohistochemical staining, if the secretion of GAs and \(\alpha\)-amylase from the cells in seed coat is very smooth, little of these molecules remains in the seed coat, making their detection impossible.

Combined the present *in situ* data and the previous immunohistochemical data together, the following explanation is possible, that active GAs synthesized in seed coat were secreted outside and received by a receptor on cellular membrane to induce \(\alpha\)-amylase that was also secreted to the integument (schematic model is shown in Fig. 5). The spatial and temporal expression patterns of InGA3ox2 and InAmy1 in seed coat support our previous explanation that GAs contribute to seed development including the development of cotyledons.

![Fig. 5](image-url)

**Fig. 5.** Spatial Comparison between the Site for InAmy1 Expression and the Localized Site of Its Product.

The left column shows the expression analysis of InAmy1 by *in situ* hybridization with paraffin-embedded sections obtained from 9-DAA developing seeds. (a), staining with the antisense probe for InAmy1; (b), control staining with the sense probe. (SC), seed coat; (IT), integument. Bar is 20\(\mu\)m. Arrows at seed coat indicate the specific staining due to the expression of InAmy1 mRNA. The right column shows the immunohistochemical analysis of the InAmy1 product with the antiserum specific for recombinant InAmy1. (c), staining with the antiserum; (d), control staining with normal serum. Arrows at integument indicate the specific staining due to the existence of InAmy1 product. (e), schematic model for a flow of InAmy1 in the developing seed of morning glory.
through the induction of α-amylase in immature seeds of \( I. \) \( nil \) in a similar process that proceeds in the germination of cereal seed.

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**References**


