cDNA Cloning and Characterization of Gibberellin-responsive Genes in Photoblastic Lettuce Seeds

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Received January 20, 1995

Two cDNA clones, cLRG5 and cLRG11, that respond to gibberellin (GA) were isolated from seeds of photoblastic lettuce (Lactuca sativa L. cv. Grand Rapids) by differential screening. Northern blot analysis indicated that the levels of LRG5 and LRG11 mRNAs were raised to slightly higher levels 10 h after the start of GA treatment and the levels were maintained at least for further 8 h, while those in the control seeds gradually decreased. Red light irradiation had effects similar to GA treatment. The cLRG5 insert encodes a putative polypeptide of 380 amino acids that is highly homologous to alcohol dehydrogenases from several higher plants. With regard to the cLRG11 insert, no homologous gene has been reported.

The germination of photoblastic lettuce (Lactuca sativa L. cv. Grand Rapids) seeds in under phytochrome-mediated photocontrol. Red light induces germination and far-red light, given after red light, cancels the effect of red light. Applied gibberellin A$_1$ (GA$_1$) as well as red light irradiation induce the germination, and the endogenous level of GA$_1$ is increased by red light irradiation. These facts indicate gibberellin (GA) is important in induction of germination of lettuce seeds.

It has been reported that several enzymes such as isocitrate lyase, $\alpha$-galactosidase, phosphatase, mannanase, acid invertase, and proteinase are activated in lettuce seeds by red light irradiation. The level of glutamine synthetase protein in the embryonic axes of lettuce seeds was also raised by red light or by GA treatment, suggesting that de novo synthesis of glutamine synthetase is closely related to seed germination. However, information is still quite limited on the molecular mechanisms of induction of lettuce seed germination. To accumulate information on genes that control lettuce seed germination, we have attempted cDNA cloning and characterization of GA-responsive genes in photoblastic lettuce seeds by differential screening in this study.

Materials and Methods

Plant material. The seeds of Grand Rapids harvested in 1992 were purchased from South Pacific Seeds in Australia. The seeds were 1 year old and had been stored at 4℃ until used. Light sources and filters were described by Toyomasu et al. To isolate RNA for construction of a cDNA library, the seeds (10 g) were incubated in the dark in a plastic box (295 mm × 220 mm, 43 mm in depth) containing 100 ml of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (1 mM, pH 6.1) with 2 × 10$^{-3}$ M GA$_3$. Three h after the start of imbibition the seeds were irradiated by far-red light for 15 min. Immediately after far-red light irradiation 40 ml of buffer was removed. The resultant seeds were incubated in the dark for 25 min. Nine h after the far-red light irradiation, the seeds [GA$_3$-+] seeds] were harvested and frozen immediately in liquid nitrogen. As a control, GA$_3$-seeds were prepared by the same method except that GA$_3$ was not contained in the incubation buffer (the far-red light irradiation 3 h after the start of imbibition was done to suppress the small percent of germination in complete darkness).

To isolate total RNA for Northern blot analysis, the seeds (0.5 g) were left to imbibe in the dark for 3 h in a Petri dish (6 cm i.d.) that contained 1 ml of MES buffer (1 mM, pH 6.1), and the following three kinds of treatments were done. The first and second groups of 3-h-imbibed seeds were transferred to Petri dishes that each contained 1.5 ml of medium, and the first group was irradiated with red light for 15 min followed by far-red light irradiation for 15 min (non-germinating conditions; R/FR seeds [control]). The second group was irradiated with red light for 15 min (germinating conditions; R seeds). The third group was transferred to a Petri dish that contained 1.5 ml of medium with GA$_3$ (2 × 10$^{-3}$ M) and then immediately irradiated by far-red light for 15 min (germinating condition; GA$_3$-treated seeds). Some of the three groups of seeds were harvested 6, 10, 14, and 18 h after each treatment.

Hosts and protocols. Escherichia coli XL1-Blue host strain and R408 interference-resistant helper phage were obtained from Stratagene (La Jolla, CA, U.S.A.). Unless otherwise indicated, protocols, buffers, media, and so forth are described by Sambrook et al.

Preparation of total and poly(A)$^+$ RNA. Total RNA was extracted from the frozen seeds by the guanidinium—CsCl method described by Sambrook et al. and poly(A)$^+$ RNA was isolated using a Dynabeads mRNA purification kit (Dynal A. S., Oslo, Norway) in accordance with the supplier’s instructions.

Construction of a cDNA library. Double stranded cDNA, synthesized as described by Gubler and Hoffman, was ligated to ZIP II vector (Stratagene). After in vitro packaging (Stratagene), the phages were plated with E. coli XL1-Blue, with a yield of 2 × 10$^7$ plaques/μg of mRNA.

Differential screening. Four pairs of replica filters of nylon membrane (Hybond-N, Amersham) were made from four plates on which approximately 1000 plaques were placed.

Each pair of membranes was treated for 3 h at 65℃ using rapid hybridization buffer (Amersham) and hybridized for 18 h at 65℃ with cDNA fragments 32P-labeled by the multiprime system (Amersham) as a probe. The filter was finally washed at 65℃ in 3 × SSC/0.1% SDS, and used to expose a X-ray film with an intensifying screen (Du Pont) at −70℃. After autoradiography, plaques that produced a stronger signal for the GA$^+$ probe than the GA$^-$ probe, were isolated, and the cDNA inserts were subcloned into a pBluescript SK$^-$ vector and were further characterized by Northern blotting.

Northern blot analysis. Total RNA (5 μg, for each lane on the gel) was

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Abbreviations: ABA, abscisic acid; ADH, alcohol dehydrogenase; GA, gibberellin; GA$_3$, gibberellin A$_3$; MES, 2-(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.
denatured and electrophoresed in a 1% agarose-2.2M formaldehyde gel. The RNA was transferred onto a nylon membrane (Hybond-N) using a standard blotting technique, treated for 3h at 65°C and hybridized with a 32P-labeled cDNA fragment for 18h at 65°C in rapid hybridization buffer (Amersham). Radioactivity was recorded on an imaging plate using a Bio-Image Analyzer (Fuji BAS2000; Fuji Photo Film, Tokyo).

DNA sequence analysis. A series of deletion mutants of a cDNA insert in pBluescript SK+ was prepared using a Kilo-Sequence Deletion Kit (Takara Shuzo Co., Ltd., Otsu, Japan) in accordance with the supplier's instructions. The nucleotides were sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems).

Results and Discussion

Isolation of cDNA clones for GA-responsive genes

We constructed a cDNA library using poly (A) RNA from seeds treated with 2 × 10⁻³ M GA3. Gibberellin A3 was used as a mimic of GA1, which is the main active GA inducing the lettuce seed germination. Two cDNA clones, cLRG5 and cLRG11, that respond to exogenous GA3 were isolated by differential screening using cDNA probes from GA(+) and GA(−) seeds. The cLRG5 and cLRG11 inserts consisted of 1,313 and 611 nucleotides, respectively.

Expression of LRG5 and LRG11 mRNAs by GA treatment or by red light irradiation

LRG5 and LRG11 mRNAs were not detectable in dry mature lettuce seeds (data not shown), but after 3h of imbibition these mRNAs in the seeds were clearly observed (Fig. 1: 0h after treatment). The mRNA levels in the GA3-treated seeds were further raised to slightly higher levels 10h after the start of GA3 treatment, and the levels were maintained at least for 8h more, while those in the control seeds decreased gradually (Fig. 1). Considering that levels of mRNAs should be interpreted in terms of their turnover rates, the GA3 treatment possibly promoted transcription of LRG5 and LRG11 and/or enhanced stability of their mRNAs. The expression pattern of LRG5 and LRG11 mRNAs in the R seeds was similar to that in the GA3-treated seeds (Fig. 1). The high levels of those mRNAs in the R seeds might be controlled by the endogenous GA1, which was increased by red light irradiation. Based on these results and the evidence that the lettuce seed germination had not been observed in the GA3-treated and R seeds until 10h and 14h after the start of each treatment, respectively, it can be suggested that the high levels of LRG5 and LRG11 mRNAs are required in advance for seed germination.

Nucleotide and deduced amino acid sequences of cLRG5 and cLRG11 inserts

The nucleotide and deduced amino acid sequences of the cLRG5 insert are shown in Fig. 2. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D44449. The cLRG5 insert contains a 1140 bp open reading frame, encoding a putative polypeptide of 380 amino acids. The amino acid sequence of the putative LRG5 polypeptide was examined for homology to sequences in the SWISS-PROT (Release 24) databases. The LRG5 polypeptide sequence had a very high homology to those of alcohol dehydrogenases (ADHs) from several species of higher plants: ADH2 (96.8% similarity, 92.1% identity), ADH3 (98.7% similarity, 92.1% identity), ADH3 (98.9% similarity, 91.8% identity) from Solanum tuberosum L. and ADH (96.6% similarity, 86.1% identity) from Fragaria ananassa Duch., and so on. The expression of ADH and its mRNA in higher plants has been known to be induced under anaerobic conditions. As the lettuce seeds are covered with hard seed coats and usually germinate in water or soil, the seeds are considered to be in rather anaerobic conditions in the initial stage of germination. Since it has been reported that ADH activity shows an early increase followed by rapid decline during the seed germination process in a number of species of higher plants, the increase in LRG5 mRNA level by GA as well as by red light might be transient and specific to an early stage of the lettuce seed germination.

The nucleotide and deduced amino acid sequences of cLRG11 insert are also shown in Fig. 3. These data will also appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D44450. The cLRG11 insert contains a 285-bp open reading frame, encoding a putative polypeptide of 95 amino acids. This cDNA sequence does not seem to be its fulllength, because based on Northern blot analysis the length of mRNA of cLRG11 is about 700 bp and the first ATG triplet in the deduced open reading frame is found at position 2–4. The cLRG11 insert probably lacks about 100 bp of the 5′ region. We have done back-screening of the cDNA library using the cLRG11 insert as a probe, but no longer cDNA insert has been obtained. No homologous sequence to cDNA insert has been obtained. No homologous sequence to the putative LRG11 polypeptide was found in the SWISS-PROT (Release 24) databases. Analysis of its function is under way.
Fig. 2. The Nucleotide and Deduced Amino Acid Sequences of cLRG5 Insert.

Fig. 3. The Nucleotide and Deduced Amino Acid Sequences of cLRG11 Insert.

Acknowledgments. We thank Professor K. Abe of Department of Applied Biological Chemistry, The University of Tokyo, and Professor Y. Komeda of Division of Biological Sciences, Hokkaido University, for helpful discussion. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 06660130) to H. Y. from the Ministry of Education, Science, and Culture of Japan.

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