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RESEARCH PAPER

Germination of photoblastic lettuce seeds is regulated via the control of endogenous physiologically active gibberellin content, rather than of gibberellin responsiveness

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Abstract

Phytochrome regulates lettuce (*Lactuca sativa* L. cv. Grand Rapids) seed germination via the control of the endogenous level of bioactive gibberellin (GA). In addition to the previously identified *LsGA20ox1*, *LsGA20ox2*, *LsGA3ox1*, *LsGA3ox2*, *LsGA2ox1*, and *LsGA2ox2*, five cDNAs were isolated from lettuce seeds: *LsCPS*, *LsKS*, *LsKO1*, *LsKO2*, and *LsKAO*. Using an *Escherichia coli* expression system and functional assays, it is shown that *LsCPS* and *LsKS* encode *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase, respectively. Using a *Pichia pastoris* system, it was found that *LsKO1* and *LsKO2* encode *ent*-kaurene oxidases and *LsKAO* encodes *ent*-kaurenoic acid oxidase. A comprehensive expression analysis of GA metabolism genes using the quantitative reverse transcription polymerase chain reaction suggested that transcripts of *LsGA3ox1* and *LsGA3ox2*, both of which encode GA 3-oxidase for GA activation, were primarily expressed in the hypocotyl end of lettuce seeds, were expressed at much lower levels than the other genes tested, and were potently up-regulated by phytochrome. Furthermore, *LsDELLA1* and *LsDELLA2* cDNAs that encode DELLA proteins, which act as negative regulators in the GA signalling pathway, were isolated from lettuce seeds. The transcript levels of these two genes were little affected by light. Lettuce seeds in which *de novo* GA biosynthesis was suppressed responded almost identically to exogenously applied GA, irrespective of the light conditions, suggesting that GA responsiveness is not significantly affected by light in lettuce seeds. It is proposed that lettuce seed germination is regulated mainly via the control of the endogenous content of bioactive GA, rather than the control of GA responsiveness.

Key words: Germination, gibberellin metabolism, gibberellin signalling, lettuce.

Introduction

Light-inducible seed germination, termed photoblastism, occurs in some higher plants and allows buried seeds to remain dormant until exhumed. This phenomenon was

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discovered in lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds using a germination assay (Borthwick *et al*., 1952). The germination of photoblastic lettuce seeds is regulated by phytochrome, which is a red (R) and far-red (FR) light receptor in plants (Butler *et al*., 1959). Red light irradiation induces the germination of lettuce seeds, and FR irradiation given after R cancels the effect of R; hence, phytochrome-induced changes in seeds are reversibly modulated by different light frequencies. The regulation of lettuce seed germination by phytochrome is thought to be mediated by gibberellin (GA).

Gibberellins are tetracyclic diterpenoid phytohormones that regulate various aspects of plant growth and development such as seed germination, leaf expansion, stem elongation, flowering, and seed development (Thomas and Hedden, 2006). Bioactive GAs are biosynthesized from geranylgeranyl diphosphate (GGDP), a common diterpenoid precursor, through several steps (Fig. 1), as reviewed by Thomas and Hedden (2006). Geranylgeranyl diphosphate is converted to the tetracyclic hydrocarbon *ent*-kaurene through *ent*-copalyl diphosphate (*ent*-CDP) by two distinct diterpene cyclases in plastids: *ent*-CDP synthase (CPS) and *ent*-kaurene synthase (KS). Furthermore, *ent*-kaurene is converted into GA_{12} via *ent*-kaurenoic acid by separate cytochrome P450 mono-oxygenases contained in the ER membrane: *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). Gibberellin A_{12} and GA_{53} (13-hydroxylated GA_{12}) are converted to GA_4 and GA_1 by cytosolic 2-oxoglutarate-dependent dioxygenases (2ODDs): GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). Gibberellin A_4 and GA_1 are the major bioactive GAs in higher plants and are deactivated by GA 2-oxidase (GA2ox)-catalysed 2-hydroxylation.

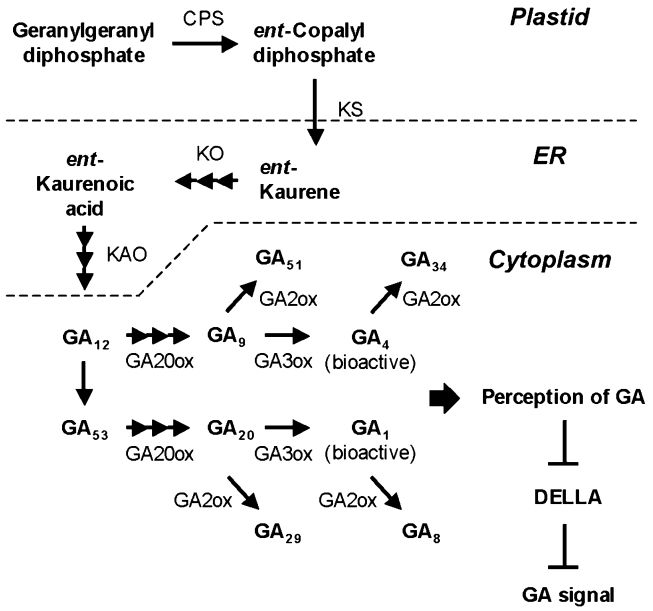

Fig. 1. Gibberellin (GA) metabolism and signal transduction in higher plants. Abbreviations: CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase; GA20ox, GA 20-oxidase; GA3ox, GA 3-oxidase; GA2ox, GA 2-oxidase.

In lettuce seeds, the application of exogenous bioactive GAs (GA_1 or GA_3, 1,2-dehydro-GA_1) mimics the effect of R light (Kahn and Goss, 1957; Ikuma and Thimann, 1960; Toyomasu *et al*., 1993). Gibberellin A_1, GA_{20}, and GA_{19} are endogenous in lettuce seeds, and phytochrome specifically regulates endogenous levels of GA_1, but not GA_{19} and GA_{20} (Toyomasu *et al*., 1993). Furthermore, the analysis and expression of six genes encoding 2ODDs (*LsGA20ox1*, *LsGA20ox2*, *LsGA3ox1*, *LsGA3ox2*, *LsGA2ox1*, and *LsGA2ox2*) showed that increases in GA_1 are caused by the phytochrome-induced up-regulation of *LsGA3ox1* (Toyomasu *et al*., 1998) and the slight down-regulation of *LsGA2ox2* (Nakaminami *et al*., 2003). The expression of these two genes is limited in the hypocotyl end of lettuce seeds (Sawada *et al*., 2008), which contains the R-perception site (Inoue and Nagashima, 1991).

Over the past decade, genetic and biochemical approaches have revealed that *Arabidopsis* seeds, for which phytochrome also regulates germination, control GA metabolism in a light-responsive manner (Yamaguchi *et al*., 1998, 2001; Seo *et al*., 2006; Yamauchi *et al*., 2007). The down-regulation of two DELLA genes, i.e. *GA-INSENSITIVE* (*GAI*) and *REPRESSOR OF GA1-3 (RGA)*, by phytochrome results in the up-regulation of GA responsiveness in *Arabidopsis* seeds (Oh *et al*., 2007). DELLA proteins are well-characterized negative regulators of GA signalling (Fig. 1) and are rapidly degraded when plants are exposed to bioactive GA (Ueguchi-Tanaka *et al*., 2007). Nevertheless, the mechanism(s) that modulate GA responsiveness in lettuce seeds remain unclear.

cDNAs encoding CPS, KS, KO, and KAO were isolated and characterized from lettuce seeds and the transcript levels of GA metabolism genes quantified using the quantitative real-time polymerase chain reaction (QRT-PCR) in imbibed whole lettuce seeds or in the hypocotyl and cotyledon ends of seeds after different light treatments. The transcript levels of GA metabolism genes in the seeds were measured under conditions in which germination was suppressed by treatment with abscisic acid (ABA) (Kahn, 1968; Sankhla and Sankhla, 1968), a phytohormone that regulates seed maturation and dormancy (Marion-Poll and Leung, 2006). To gain a better understanding of how DELLA genes regulate GA responsiveness in lettuce seeds, cDNAs encoding DELLA proteins were isolated and characterized, and germination assays were carried out in the presence of various concentrations of GA_3.

Materials and methods

Light sources and plant materials

Red (6.5 W m^{-2}) and FR (5 W m^{-2}) light were used as described previously (Toyomasu *et al*., 1993). Lettuce seeds (*Lactuca sativa*

L. cv. Grand Rapids) similar to those used in previous studies (Sawada *et al*., 2008) were stored at 4 °C with silica gel until needed. For each treatment, 50 mg of seeds (approximately 50 seeds) were incubated according to the method of Nakaminami *et al*. (2003). Seeds were exposed to one of three light treatments 3 h after the start of imbibition: irradiation with FR (FR treatment); FR followed by R (FR/R treatment); and FR followed by R and FR (FR/R/FR treatment). Each light irradiation lasted 5 min. Seeds imbibed in the dark for 3 h were also harvested immediately prior to light treatment (0 h). After light treatment, the seeds were incubated in the dark at 25 °C for 2, 4, and 6 h. The seeds were then frozen in liquid nitrogen. The application of 0.1 mM ABA to the seeds was performed by buffer exchange before light treatment. For dark incubations, seeds were handled under a dim green safety light.

Molecular cloning

The design of the degenerate primers used to clone *CPS*, *KS*, *KO*, *KAO*, and *DELLA* was based on the respective conserved amino acid regions among sequences from other plant species (see Supplementary Table S1 at *JXB* online). Template cDNA derived from lettuce seeds (Toyomasu *et al*., 1998) was used for PCR. The 50 µl reaction mixture contained 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 µM of each primer, and 2.5 units of Expand HF (Roche Diagnostics, Indianapolis, IN, USA). The samples were heated to 94 °C for 2 min, then subjected to 40 cycles at 94 °C for 1 min, 40 °C (*CPS*) or 45 °C (*KS*, *KO*, *KAO*, and *DELLA*) for 1 min, and 72 °C for 1 min, followed by a final extension for 7 min. Fragments obtained by PCR were subcloned using a pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyser (Applied Biosystems). Rapid amplification of cDNA ends (RACE) was performed using the methods of Toyomasu *et al*. (1998). Homology database searches were performed by BLAST analysis (http://www.ncbi.nlm.nih.gov/).

Functional analysis of LsCPS *and* LsKS *(diterpene cyclases)*

The coding regions of *LsCPS* and *LsKS* cDNAs were amplified by RT-PCR using primers (see Supplementary Table S2 at *JXB* online) with the proper restriction enzyme sites for subcloning into pGEX-4T-3 vector (GE Healthcare, Piscataway, NJ, USA). The preparation of plasmids, heterologous expression in *Escherichia coli*, extraction and purification of recombinant enzymes, and enzyme assays were performed using the method described by Otomo *et al*. (2004*b*) for *LsCPS* and by Otomo *et al*. (2004*a*) for *LsKS*. The reaction products were detected and analysed by gas chromatography–mass spectrometry (GC–MS) using an Agilent 6890N GC-5973N MSD mass selective detector system (ionization voltage 70 eV) fitted with a fused silica chemically bonded capillary column DB-WAX (0.25 mm diameter, 60 m length, and 0.25 µm film thickness; J&W Scientific, Folson, CA). Samples were injected into the column at 250 °C in the splitless mode. After isothermal hold at 80 °C for 2 min, the column temperature was increased by 5 °C min^{-1} to 250 °C, with an isothermal hold at 250 °C for 15 min. The flow rate of the helium carrier gas was 1 ml min^{-1}.

Functional analysis of LsKO *and* LsKAO *(P450 mono-oxygenases)*

The coding regions of *LsKO1*, *LsKO2*, and *LsKAO* were amplified using specific primer sequences (see Supplementary Table S2 at *JXB* online). Each fragment was ligated into a yeast expression vector (pPICZ) using the appropriate restriction enzyme sites. Recombinant enzymes were co-expressed by the addition of methanol in each yeast (*Pichia pastoris* X-33) transformant harbouring both the NADPH-P450 reductase gene and the targeted P450 gene. The microsome fraction was prepared by ultracentrifugation (100 000 *g*, 4 °C, 1 h) and was then incubated with the proper substrate in the presence of 5 mM NADPH. The sample was analysed by GC-MS (JEOL JMS-Bu25) equipped with a DB-5 capillary column (0.25 mm diameter, 15 m length, and 0.25 µm film thickness; J&W Scientific). For the analysis of KO products, samples were derivatized with diazomethane and injected into the GC–MS instrument. After an isothermal hold at 80 °C for 1 min, the column temperature was increased by 30 °C min^{-1} to 200 °C and then by 5 °C min^{-1} to 280 °C. For the analysis of KAO products, derivatized samples were analysed by GC-MS as described by Kawaide *et al*. (1995).

Functional analysis of lettuce DELLA

The coding regions of *LsDELLA1* and *LsDELLA2* cDNA were amplified by RT–PCR with specific primers (see Supplementary Table S2 at *JXB* online) that had suitable restriction enzyme sites at the 5′ end of each primer. The products obtained by PCR were subcloned into pGEX-4T-3 vector. The preparation of plasmids, heterologous expression in *E. coli*, and extraction and purification of recombinant proteins were performed using the methods of Otomo *et al*. (2004*b*). Experiments to probe the interactions of AtGID1c-GA with LsDELLA were performed using the methods described previously (Ueguchi-Tanaka *et al*., 2005; Nakajima *et al*., 2006). The [1, 2, 16, 17-$^3H_4$]-16, 17-dihydro–GA_4 was used as labelled GA, and unlabelled GA_4 was used as competitor to test for non-specific binding. For the glutathione *S*-transferase (GST) pull-down assay, GST-LsDELLA or GST (negative control) bound to glutathione-Sepharose 4B beads (GE Healthcare) was mixed with crude Trx-AtGID1c or Trx (negative control) in 270 µl of binding buffer (PBS, pH 7.0) with or without 0.1 mM GA_4 and incubated at 25 °C for 30 min. The beads were then sedimented by centrifugation and washed with binding buffer. The beads were resuspended in 15 µl of elution buffer (50 mM TRIS-HCl, pH 8.0, containing 10 mM glutathione) and centrifuged. Proteins in the supernatant were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB).

Quantitative real-time PCR

Total RNA was extracted from frozen samples using an RNAqueous column with the Plant RNA Isolation Aid (Ambion, Austion, TX, USA). For the expression analysis of half-cut seeds, frozen seeds were divided into two parts on dry ice using a razor. Complementary DNA was prepared from 1 µg of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative RT-PCR using SYBR Green I was carried out on a Thermal Cycler Dice Real Time System (TP800, Takara bio, Otsu, Japan) as described previously (Bustin, 2000; Sawada *et al*., 2008) using specific primers (see Supplementary Table S3 at *JXB* online). The means of two or three replicates were normalized using 18S rRNA internal controls.

Germination assay

Twenty decoated lettuce seeds were incubated in the dark at 25 °C with 50 µM uniconazol-P and various concentrations of GA_3. The decoated lettuce seeds did not contain seed coats. At 2 h after the start of imbibition, seeds were exposed to R light (3.3 W m^{-2}) for 3 min. The seeds were then incubated in the dark for 24 h, and the germination frequency was recorded. Germination of decoated seeds was determined by the protrusion of root tip from remained endosperm layer. Five independent experiments were performed.

Accession numbers

Sequence data were deposited with the GenBank/EMBL data libraries under accession numbers AB031204 (LsCPS), AB031205 (LsKS), AB370235 (LsKO1), AB370236 (LsKO2), AB370237 (LsKAO), AB370238 (LsDELLA1), and AB370240 (LsDELLA2).

Results

Cloning of cDNAs encoding CPS and KS in lettuce

To clone *CPS* and *KS*, degenerate primers were designed based on the conserved amino acid sequences of other plant CPSs and KSs, respectively. Complementary DNA fragments of the expected size of approximately 810 bp (*CPS*) and 510 bp (*KS*) were amplified from the templates derived from R light-treated seeds by RT–PCR using the degenerate primers. Subcloned fragments were analysed according to their sequence similarity with other plant cyclases, indicating that one *CPS*-like fragment and one *KS*-like fragment were present. RACE was performed to determine each full-length cDNA sequence using gene-specific primers. End-to-end PCR was then performed using 5′- and 3′-end primers. The clones were named *LsCPS* and *LsKS*. The predicted coding regions of *LsCPS* and *LsKS* were 2400 and 2367 bp, encoding 799 and 788 amino acid residues, respectively. The predicted amino acid sequence of LsCPS showed 74% homology with *Stevia rebaudiana* CPS (AF034545), and that of LsKS showed 70% homology with *Stevia* KS (AF097310).

The enzymatic functions of LsCPS and LsKS were characterized in *in vitro* assays using recombinant proteins expressed in bacteria. The coding regions of *LsCPS* and *LsKS* cDNA were subcloned into bacterial expression vectors, and the recombinant enzymes were fused with GST at the N terminus. The GST fusion proteins were purified by affinity chromatography. GST-LsCPS was then incubated with GGDP. After dephosphorylation using bacterial alkaline phosphatase, the reaction product was identified by full-scan GC-MS as the alcohol derivative. The retention time and mass spectrum of the dephosphorylated product were identical to those of authentic *ent*-copalol (see Supplementary Table S4 at *JXB* online). Recombinant GST-LsKS was incubated with GGDP and GST-LsCPS, and the product was analysed by GC-MS. A peak with a retention time and mass spectrum identical to authentic *ent*-kaurene was observed (see Supplementary Table S4 at *JXB* online). Collectively, these analyses showed that the GST-LsCPS product *ent*-CDP was converted into *ent*-kaurene by GST-LsKS and suggested that *LsCPS* and *LsKS* encode CPS and KS, respectively.

Cloning of cDNAs encoding KO and KAO in lettuce

To clone *KO* and *KAO* homologues from lettuce, RT-PCR was performed with specific degenerate primers, which were designed based on the conserved amino acid sequences of other plant KOs and KAOs, respectively. Bands were amplified at approximately 420 bp (*KO*) and 440 bp (*KAO*). Nucleotide sequencing of each subcloned fragment indicated that two *KO*-like fragments (*LsKO1* and *LsKO2*) and one *KAO*-like fragment (*LsKAO*) were obtained. Each full-length sequence was determined by RACE. The predicted coding regions of *LsKO1*, *LsKO2*, and *LsKAO* were 1536, 1539, and 1482 bp, encoding 511, 512, and 493 amino acid residues, respectively. These three clones were named *LsKO1*, *LsKO2*, and *LsKAO*. The predicted coding regions of these cDNAs were highly homologous to those from other plant species and were 71% (LsKO1/*Stevia* KO: AY364317), 78% (LsKO2/*Stevia* KO), and 65% (LsKAO/*Arabidopsis* KAO1: NM100394) similar in amino acid sequence.

Assays to elucidate the functions of the translated gene products were carried out *in vitro* using a yeast expression system based on *Pichia pastoris*. Each cDNA was expressed in yeast harbouring an *Arabidopsis* P450 reductase to yield the recombinant protein. *ent*-Kaurene and *ent*-kaurenoic acid were used as substrates in enzyme assays for KO and KAO, respectively, using yeast microsomal fractions. The reaction products were identified by GC–MS as methyl ester derivatives. *ent*-Kaurenoic acid and GA_{12} were identified as major products converted from *ent*-kaurene by LsKO1 and LsKO2 and from *ent*-kaurenoic acid by LsKAO, respectively (see Supplementary Table S5 at *JXB* online). These results suggest that *LsKO1* and *LsKO2* encode KO and that *LsKAO* encodes KAO.

Gene expression during germination

Three types of pulsed light treatment were used in the germination studies. The FR light treatment was used to suppress germination in the dark and thus served as a negative control for germination. The FR/R light treatment was used to induce germination. The FR/R/FR light treatment was performed to confirm the regulation of germination by phytochrome. In the FR/R treatment group, the radicle appeared 8 h after the light treatment (Fig. 2A). To determine the expression levels of GA metabolism genes in the lettuce seeds, QRT-PCR analyses were performed (Fig. 2B). *LsGA2ox1* transcripts were the most abundant of the GA metabolic genes in the imbibed seeds; the expression levels of two *LsGA3ox* genes were much lower than those of other GA metabolic genes. The transcript level of *LsKAO* in FR/R-treated seeds increased slightly compared with that in FR- and FR/R/FR-treated seeds until 6 h. The transcript levels of *LsCPS*, *LsKS*, *LsKO1*, and *LsKO2* were not affected by light treatment. Consistent with previous results (Toyomasu *et al.*, 1998), the transcript level of *LsGA20ox2* decreased and that of *LsGA3ox1* increased robustly after FR/R treatment. The expression of *LsGA20ox1* increased very slightly after FR/R treatment. Similar to *LsGA3ox1*, the transcript level of

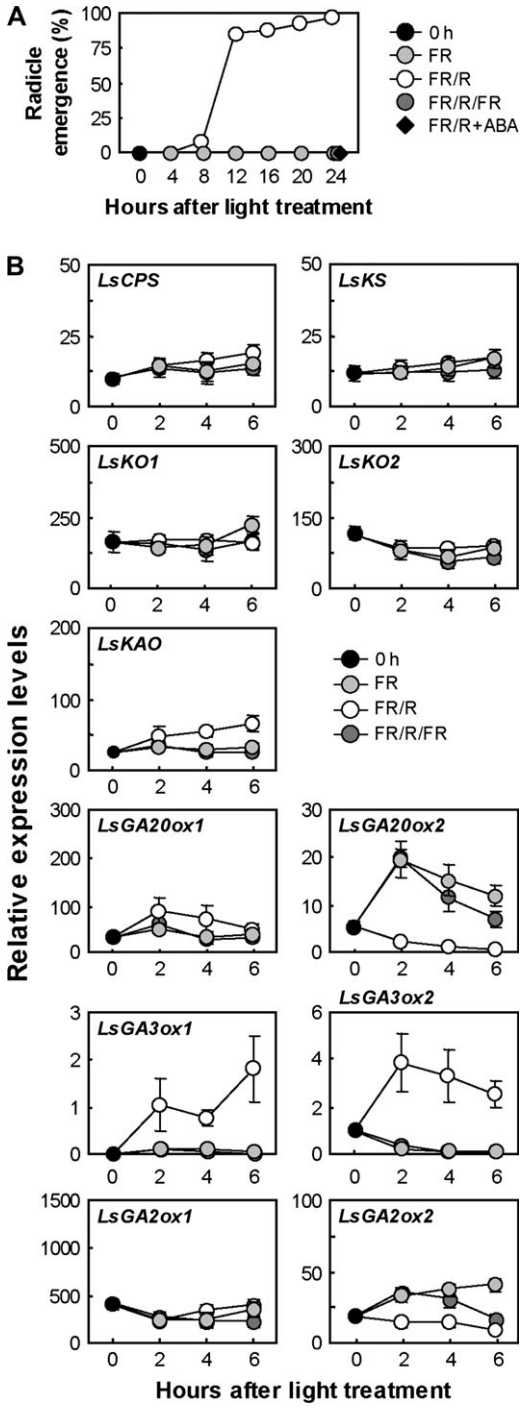

Fig. 2. Changes in the transcript levels of gibberellin (GA) metabolism genes in imbibed lettuce seeds after various light treatments. (A) Time-course of lettuce seed germination after light treatment. Zero (0) h indicates seeds imbibed for 3 h in the dark that received no light treatment. FR, FR/R, and FR/R/FR indicate seeds treated with far-red light, far-red followed by red light, and far-red followed by red and then far-red light, respectively. FR/R+ABA indicates seeds treated with far-red followed by red light and 0.1 mM ABA. Triplicate experiments were performed, and means with standard errors are shown. (B) Expression levels of GA metabolism genes after light treatment. The expression levels of these genes were analysed by QRT-PCR. The results were normalized to the expression of 18S rRNA (internal control); the expression levels of all genes examined are given relative to the reference value of the transcript level of *LsGA3ox2* at 0 h, set to 1. Three independent experiments were performed, and means with standard errors are shown.

LsGA3ox2 increased considerably after FR/R treatment. The effects of R light on the expression of *LsGA20ox2*, *LsGA3ox1*, and *LsGA3ox2* were abolished by successive FR irradiation (FR/R/FR). The expression of *LsGA2ox1* was not affected by light treatment, and the transcript level of *LsGA2ox2* in FR/R seeds was lower than that in FR seeds, similar to previous results (Nakaminami *et al*., 2003). Nevertheless, the transcript level of *LsGA2ox2* in FR-treated seeds was different from that in FR/R/FR-treated seeds 6 h after light treatment. Nakaminami *et al*. (2003) showed that the regulation of *LsGA2ox2* expression is photoreversible using one-point expression analysis. Non-photoreversible regulation of *LsGA2ox2* was observed using time-course expression analysis. These results suggest that the regulatory mechanism of *LsGA2ox2* expression is somewhat different from that of *LsGA20ox2*, *LsGA3ox1*, and *LsGA3ox2*. Hence, the comprehensive expression analysis of GA metabolic genes strongly suggests that the robust up-regulation of *LsGA3ox1* and *LsGA3ox2* and down-regulation of *LsGA2ox2* is primarily responsible for GA_1 augmentation after FR/R treatment in imbibed lettuce seeds (Toyomasu *et al*., 1993), although we could not completely remove the possibility that unidentified paralogues of *GA3ox* and *GA2ox* in lettuce are involved.

Sawada *et al*. (2008) detected *LsGA3ox1* and *LsGA2ox2* transcripts mainly on the hypocotyl end of the seed during germination. To examine the expression of other GA metabolic genes on both ends of lettuce seeds, the transcripts of GA metabolic genes on the cotyledon and hypocotyl ends of lettuce seeds were studied using QRT-PCR (Fig. 3B) following the methods of Sawada *et al*. (2008). The cotyledon end of the seed is composed of the cotyledons, fruit wall, seed coat, and endosperm; the hypocotyl end of the seed includes the hypocotyl, root apical meristem, shoot apical meristem, and part of the cotyledons, fruit wall, seed coat, and endosperm (Fig. 3A). The transcript levels of *LsCPS*, *LsKO1*, and *LsGA20ox1* in the hypocotyl end were higher than those in the cotyledon end, whereas the transcript levels of *LsKS*, *LsKO2*, *LsGA20ox2*, and *LsGA2ox1* were similar in the hypocotyl end to those in the cotyledon end. The expression of *LsGA20ox1* was slightly up-regulated by FR/R treatment in the cotyledon end, but not in the hypocotyl end, whereas the expression of *LsGA20ox2* was down-regulated in both ends. The very slight increase in *LsGA20ox1* expression in whole seeds (Fig. 2B) may have been caused by increases in the cotyledon end. Transcripts of *LsKAO* and *LsGA3ox2* were also detected mainly on the hypocotyl end, rather than the cotyledon end. The expression of *LsGA3ox1* and *LsGA3ox2* was dramatically up-regulated and that of *LsGA2ox2* was down-regulated, whereas the expression of *LsKAO* was slightly up-regulated in the hypocotyl end after FR/R treatment compared to FR treatment. These patterns of gene

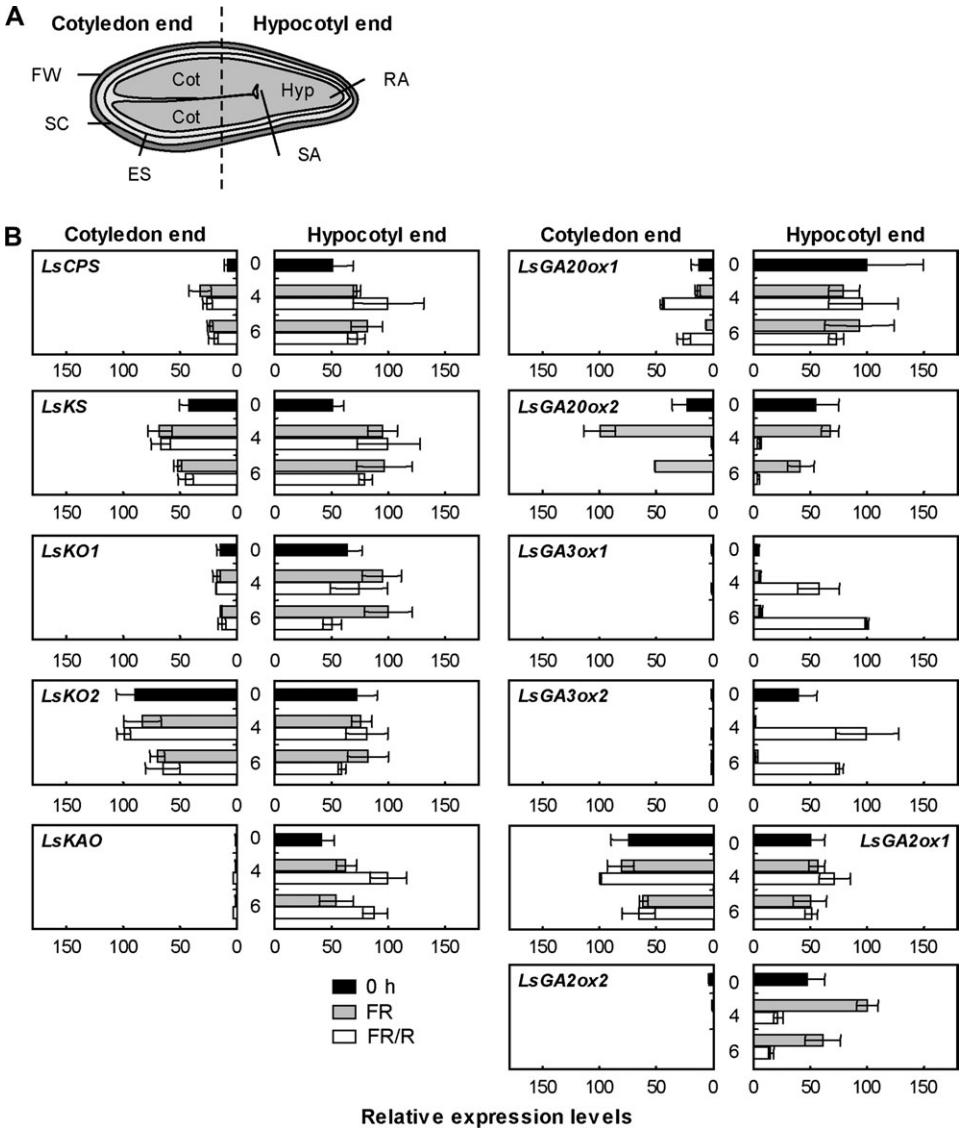

Fig. 3. Changes in the transcript levels of gibberellin (GA) metabolism genes in the cotyledon end and hypocotyl end of lettuce seeds. (A) Frozen seeds were divided into two parts: cotyledon end, including the cotyledons (Cot), fruit wall (FW), seed coat (SC), and endosperm (ES); and the hypocotyl end, including the hypocotyl (Hyp), root apical meristem (RAM), shoot apical meristem (SAM), and part of the Cot, FW, SC, and ES. (B) Expression levels of GA metabolism genes after light treatment, determined using QRT-PCR. See Fig. 2 for light treatments. The results were normalized to the expression of 18S rRNA (internal control), and the highest value was set to 100. Two independent experiments were performed, and means with standard errors are shown.

expression in the hypocotyl end of the seed agreed with those in whole seeds. It is noteworthy that transcripts that are regarded as involved in the up-regulation of GA_1 content after FR/R treatment, such as *LsGA3ox1*, *LsGA3ox2*, and *LsGA2ox2*, are mainly localized in the hypocotyl end of the seed.

Effects of ABA treatment on the expression of GA metabolism genes during seed germination

Lettuce seed germination induced by R light or by GA treatment is inhibited by the application of ABA (Kahn, 1968; Sankhla and Sankhla, 1968). Under our experimental conditions, lettuce seed germination in FR/R-treated seeds was completely suppressed by the application of 0.1 mM ABA (Fig. 2A). To examine whether GA metabolism genes are affected by exogenously applied ABA, QRT-PCR was performed on 11 GA metabolism genes using ABA-treated seeds. Among the genes of which the expression is regulated mainly by light, ABA treatment slightly down-regulated the expression of *LsGA3ox1* and slightly up-regulated that of *LsGA2ox2*, whereas the expression of *LsGA20ox2* and *LsGA3ox2* was not affected (see FR/R and FR/R+ABA seeds in Fig. 4A). The expression of the other seven genes that were examined was not markedly affected by ABA (data not shown). These results were confirmed by QRT-PCR using half-cut

seeds. The up-regulation of *LsGA3ox1* and the down-regulation of *LsGA2ox2* by FR/R treatment in the hypocotyl end of the seed were partly abolished by ABA treatment, whereas the up-regulation of *LsGA3ox2* by FR/R treatment was unaffected (Fig. 4B).

Cloning of cDNA encoding DELLA proteins in lettuce

To examine the expression levels of *DELLA* genes in lettuce seeds, cDNA that encodes DELLA proteins was isolated. One set of degenerate primers was designed based on the conserved amino acid sequences of DELLA proteins in *Arabidopsis* and rice (*Oryza sativa* L.). A band of the expected size (~590 bp) was amplified from the cDNA template derived from R-treated seeds by RT-PCR using the degenerate primers. Nucleotide sequence analyses indicated the presence of two different *DELLA*-like fragments. The two fragments were named *LsDELLA1* and *LsDELLA2*. The RACE and end-to-end PCR analyses indicated that the predicted coding regions of *LsDELLA1* and *LsDELLA2* were 1710 bp and 1773 bp, encoding 569 and 590 amino acid residues, respectively. The amino acid sequences of LsDELLA1 and LsDELLA2 showed 62% and 61% similarity with that of *Arabidopsis* GAI (NM101361), respectively.

Gibberellin insensitive dwarf 1 (*GID1*) encodes a soluble GA receptor in rice (Ueguchi-Tanaka *et al*., 2005); three orthologues (*AtGID1a*, *AtGID1b*, and *AtGID1c*) were identified in *Arabidopsis* (Nakajima *et al*., 2006). It has been shown that GID1 binds directly to DELLA in the presence of bioactive GA. To determine whether LsDELLA1 and LsDELLA2 also bind GID1, pull-down assays were performed using recombinant LsDELLA and AtGID1c. Each coding region of *LsDELLA1* and *LsDELLA2* cDNA was subcloned into a bacterial expression vector; recombinant proteins that were fused with GST at the N terminus were purified. Crude recombinant AtGID1c fused with thioredoxin (TRX) at the N terminus was also obtained. The affinity-purified GST-LsDELLA proteins or GST alone (negative control) were incubated with crude TRX-AtGID1c or TRX alone (negative control) in the presence or absence of GA_4. TRX-AtGID1c interacted with GST-LsDELLAs, which were precipitated by glutathione resin, in the presence of GA_4 (Fig. 5A). To examine whether LsDELLAs increase the GA-binding activity of AtGID1c, an *in vitro* binding assay was performed using labelled GA in accordance with protocols used in rice and *Arabidopsis* (Ueguchi-Tanaka *et al*., 2005; Nakajima *et al*., 2006). After mixing labelled 16,17-dihydro-GA_4 and AtGID1c, each affinity-purified LsDELLA was added to the mixture, and its GA-binding activity was measured. The activity of AtGID1c increased after the addition of not only *Arabidopsis* GAI (positive control) but also LsDELLAs (Fig. 5B). These results suggest that *LsDELLA1* and *LsDELLA2* encode DELLA proteins.

GA responsiveness in imbibed lettuce seeds

Red light treatment up-regulates the GA responsiveness of *Arabidopsis* seeds through PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5), a helix-loop-helix

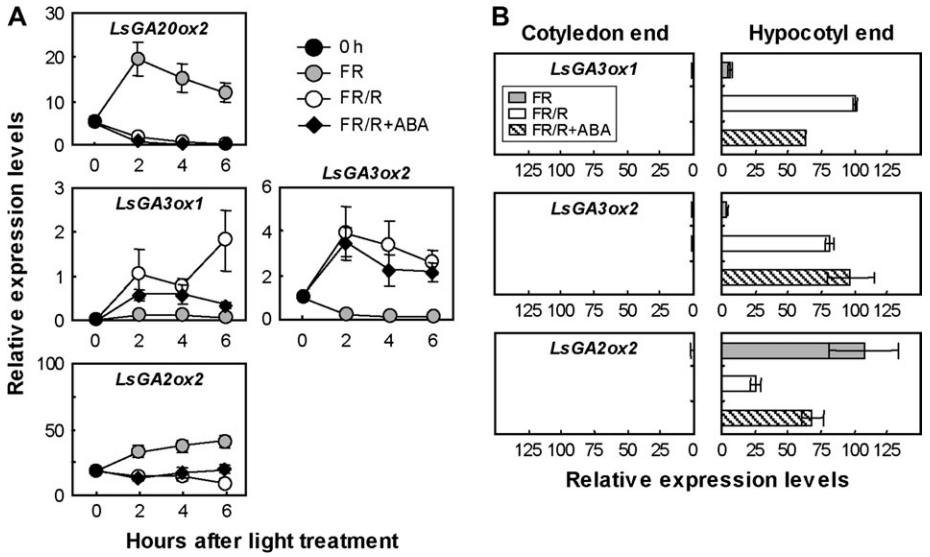

Fig. 4. Effects of abscisic acid (ABA) treatment on the expression levels of gibberellin (GA) metabolism genes in imbibed lettuce seeds. (A) The expression levels of the genes were analysed by QRT-PCR. The results were normalized to the expression of 18S rRNA (internal control), and the expression levels of all genes examined are given relative to the reference value of the transcript level of *LsGA3ox2* at 0 h, set to 1. Three independent experiments were performed, and means with standard errors are shown. (B) Expression analysis using seeds that had been cut in half. The results were normalized to the expression of 18S rRNA (internal control), and the highest value was set to 100. Two independent experiments were performed, and means with standard errors are shown.

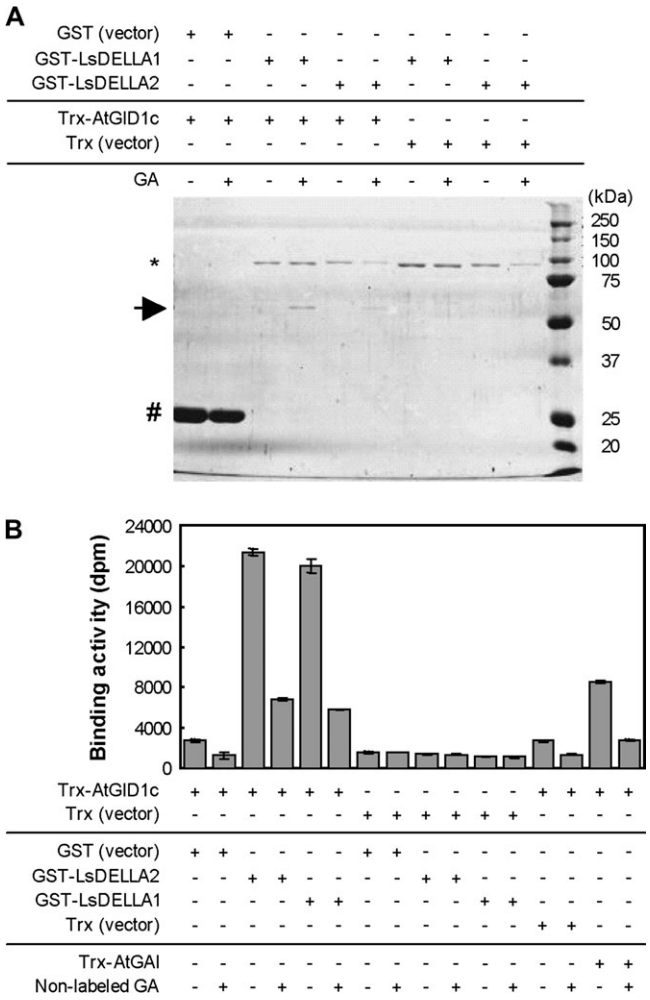

Fig. 5. *In vitro* functions of two LsDELLA proteins. (A) Trx-AtGID1c was pulled down by GST-LsDELLAs in a GA-dependent manner. The pull-down assay was performed using purified LsDELLA and crude AtGID1c. Trx-AtGID1c or Trx were incubated with GST-LsDELLA or GST in the presence or absence of 0.1 mM GA_4. The protein samples were separated by 10% SDS-PAGE and stained using CBB. The asterisk, arrowhead, and # indicate GST-DELLA, Trx-AtGID1c, and GST, respectively. (B) Increase in the GA-binding activity of AtGID1c by LsDELLAs *in vitro*. GST and crude Trx were used as negative controls. Three independent experiments were performed, and means with standard deviations are shown.

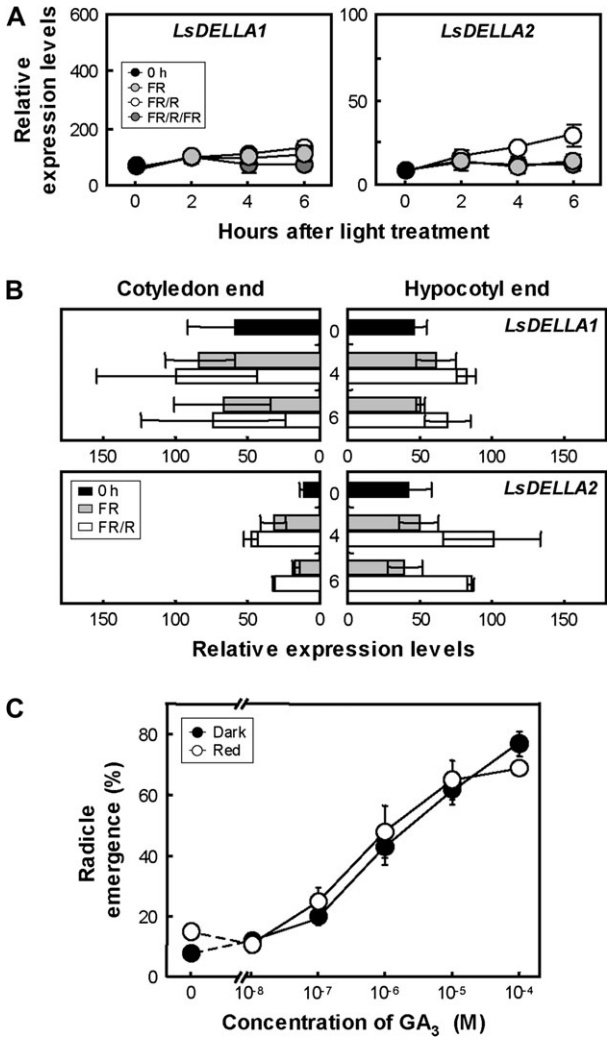

Fig. 6. Expression of *LsDELLA1* and *LsDELLA2* during germination, and gibberellin (GA) responsiveness in lettuce seeds. (A) Expression levels of *LsDELLA1* and *LsDELLA2* after light treatment. The expression analysis was carried out by QRT-PCR. See Fig. 2 for light treatments. The results were normalized to the expression of 18S rRNA (internal control), and the expression levels of all genes examined are given relative to the reference value of the transcript level of *LsGA3ox2* at 0 h, set to 1. Three independent experiments were performed, and means with standard errors are shown. (B) Expression levels of *LsDELLA1* and *DELLA2* in the cotyledon end and the hypocotyl end of lettuce seeds after light treatment. The results were normalized to the expression of 18S rRNA (internal control), and the highest value was set to 100. Two independent experiments were performed, and means with standard errors are shown. (C) Germination frequency of lettuce seeds in the presence of a GA biosynthesis inhibitor and various concentrations of GA_3. Five sets of 20 decoated lettuce seeds were incubated in the dark at 25 °C in medium containing 50 μM uniconazol-P and various concentrations of GA_3. After red light treatment, seeds were incubated at 25 °C in the dark for 24 h and the germination frequency was recorded. Means with standard errors are shown.

protein. In *Arabidopsis* seeds, the expression of two *DELLA* genes, i.e. *RGA* and *GAI*, is down-regulated by phytochrome (Oh *et al*., 2007). To determine if similar phenomena occur in lettuce seeds, *LsDELLA* genes were examined by QRT-PCR after different light treatments (Fig. 6A). The transcript level of *LsDELLA1* was much higher than that of *LsDELLA2* and was unaffected by light treatment. The transcript level of *LsDELLA2* was slightly increased after FR/R treatment. Furthermore, expression analysis using half-cut seeds showed that the two *LsDELLA* transcripts accumulated almost equally on both the cotyledon and the hypocotyl ends of the seed (Fig. 6B). Slight increases in *LsDELLA2* transcripts were observed on both ends of the seed after FR/R treatment (Fig. 6B). These results suggest that the expression of *LsDELLA* genes is not markedly affected by light treatment and that their expression patterns in lettuce seeds differ from those of *RGA* and *GAI* in *Arabidopsis* (Oh *et al*., 2007).

These collective observations led us to posit that GA responsiveness is not altered by light treatment in lettuce seeds. Therefore, the germination of decoated lettuce seeds was examined in which *de novo* GA biosynthesis was suppressed by uniconazole-P (GA biosynthesis inhibitor) in the presence of various concentrations of GA_3. Notably, GA_3 is not metabolized by GA2ox (Nakayama *et al*., 1990; Oh *et al*., 2007). Uniconazole-P treatment suppressed radicle emergence in seeds treated with R light and in non-irradiated seeds (Fig. 6C). The germination frequency of decoated lettuce seeds after R treatment increased in a manner similar to that of the GA dose response in non-irradiated seeds (Fig. 6C). These results suggest that GA responsiveness is not markedly altered by R light in lettuce seeds.

Discussion

Since the 1950s, many studies have explored the regulatory mechanisms of photoblastic lettuce seed germination, including biochemical, physical, and physiological studies (see many references that have been published). Of the regulatory mechanisms, the role of GAs and ABA in the regulation of lettuce seed germination is critical. Using biochemical and expression analyses, the control of endogenous GA levels has been shown to be critical for the germination of lettuce seeds after R irradiation (Toyomasu *et al*., 1993, 1998; Nakaminami *et al*., 2003; Sawada *et al*., 2008). This view has also been reported for *Arabidopsis* seeds (Yamaguchi *et al*., 1998, 2001; Ogawa *et al*., 2003; Seo *et al*., 2006; Yamauchi *et al*., 2007). Three classes of GA metabolism enzymes have been identified in several higher plant species, including *Arabidopsis* and rice (Thomas and Hedden, 2006). These include diterpene cyclases (CPS and KS), P450 monooxygenases (KO and KAO), and 2ODDs (GA20ox and GA3ox for GA biosynthesis and GA2ox for GA deactivation). In addition to *LsGA20ox1*, *LsGA20ox2*, *LsGA3ox1*, *LsGA3ox2*, *LsGA2ox1*, and *LsGA2ox2* (Toyomasu *et al*., 1998; Nakaminami *et al*., 2003), cDNAs that encode CPS, KS, KO, and KAO were isolated and comprehensive expression analyses were performed to clarify the regulation of GA metabolism in lettuce seeds. To our knowledge, no detailed quantification by QRT-PCR of the transcripts of overall GA metabolism genes in imbibed seeds has yet been reported.

Our comprehensive expression analysis using QRT-PCR showed that the transcript levels of *LsGA3ox1* and *LsGA3ox2* were much lower than those of the other genes tested and were dramatically up-regulated by R irradiation (Fig. 2B). In imbibed lettuce seeds, endogenous levels of GA_1 were much lower than those of GA_{19} (approximately 1/10th) and GA_{20} (approximately 1/100th), both of which are GA_1 precursors (Toyomasu *et al*., 1993). These data suggest that GA_1 levels are strictly regulated by GA3ox in lettuce seeds and that higher levels of GA_{19} and GA_{20} could be a result of the greater expression of other GA biosynthetic genes. The expression of *LsKAO*, *LsGA20ox1*, *LsGA3ox1*, and *LsGA3ox2* was suggested to be primarily responsible for GA_1 biosynthesis after FR/R treatment, whereas the physiological role of the down-regulation of *LsGA20ox2* expression by R light remains unclear (Fig. 2B). Transcripts of *LsKAO*, *LsGA3ox1*, and *LsGA3ox2* were mainly detected in the hypocotyl end of the seed (Fig. 3B). The *ent*-kaurenoic acid oxidase converts the *ent*-kaurene skeleton into the *ent*-gibberellane skeleton, resulting in the production of GA_{12}, which is the first step in the formation of GA. Generally, the step catalysed by CPS (conversion of GGDP into *ent*-CDP) is regarded as the first committed step in GA biosynthesis. However, *ent*-CDP is converted into *ent*-pimaradiene-related phytoalexins in rice (Cho *et al*., 2004; Otomo *et al*., 2004*a*), and *ent*-kaurenoic acid is converted into stevioside in *Stevia* (Tanina *et al*., 2006). Little is known about the detailed metabolic pathways of diterpenoids in imbibed lettuce seeds; regardless, KAO most probably catalyses the first step of GA biosynthesis. This strongly suggests that GA is mainly synthesized *de novo* in the hypocotyl end of germinating lettuce seeds, although the localization of accumulated GAs in lettuce seeds remains unclear.

The transcript of *LsGA2ox1*, a GA deactivation gene that was not regulated by light, was detected in both ends of lettuce seeds (Figs 2B, 3B). Another GA deactivation gene that was detected mainly in the hypocotyl end, *LsGA2ox2*, was down-regulated by R light treatment (Figs 2B, 3B). Nakaminami *et al*. (2003) found that recombinant LsGA2ox1 hydroxylated C_2 of GA_{20}, as well as that of GA_1, and recombinant LsGA2ox2 hydoxylated C_2 of GA_{20} *in vitro*, although the substrate specificity of both enzymes was not confirmed *in vivo*. *LsGA2ox1* may be responsible for the constitutive metabolism of GA_1 and GA_{20} in imbibed seeds; the down-regulation of *LsGA2ox2* in the hypocotyl end of seeds as a result of R irradiation could contribute to GA_1 increment.

The patterns of regulation by light of the two *GA3ox* genes in lettuce are similar to those in *Arabidopsis* (Yamaguchi *et al*., 1998). In *Arabidopsis* seeds, the regulation of *AtGA3ox1*/*GA4* and *AtGA3ox2*/*GA4H* gene expression differs; *AtGA3ox2*/*GA4H* expression is regulated by PHYB and is not subject to feedback regulation by the GA signal, whereas *AtGA3ox1*/*GA4* expression is regulated by other phytochromes and is subject to feedback regulation (Yamaguchi *et al*., 1998). Transcripts of both of the genes were detected in the cortex of R-treated seeds (Yamaguchi *et al*., 2001). The regulation of *LsGA3ox1* and *LsGA3ox2*, both of which were detected in the hypocotyl end of lettuce seeds (Fig. 3B), differed in terms of the involvement of ABA. ABA treatment

down-regulated the expression of *LsGA3ox1*; however, *LsGA3ox2* expression was not down-regulated, even in ABA-treated seeds that did not germinate (Fig. 4). In *Arabidopsis*, both *AtGA3ox* genes were down-regulated in the ABA-overaccumulating mutant *cyp707a2-1* seeds (Seo *et al*., 2006). Furthermore, ABA deficiency (*nced6-1*, *aba2-2*, *aao3-4*) enhances the germination frequency, increases levels of endogenous physiologically active GA (GA_4), and augments the transcript levels of *AtGA3ox1* and *AtGA3ox2* in FR-treated seeds (Seo *et al*., 2006). Thus, Seo *et al*. (2006) suggested that ABA affects GA biosynthesis during *Arabidopsis* seed germination. Conversely, GA affects ABA levels by controlling the expression of ABA metabolism genes in *Arabidopsis* (Oh *et al*., 2007). Sawada *et al*. (2008) reported that endogenous levels of ABA decrease after the induction of germination by GA treatment via the down-regulation of *LsNCED4*. It appears that GA and ABA diametrically oppose each other in both *Arabidopsis* and lettuce seeds, although detailed regulation patterns of the targeted genes differ. In future studies, the measurement of endogenous GA levels after ABA treatment could provide more information on the interaction between GA and ABA in lettuce seeds.

Light alters both GA responsiveness and GA metabolism in *Arabidopsis* seeds. Specifically, PIL5 regulates GA responsiveness by directly binding to the promoters of *GAI* and *RGA* in *Arabidopsis* seeds (Oh *et al*., 2007). The *Arabidopsis* genome contains five *DELLA* genes (*GAI*, *RGA*, *RGL1*, *RGL2*, and *RGL3*) and the rice genome contains one (*SLR1*) (Ueguchi-Tanaka *et al*., 2007). Two *DELLA* genes were isolated from lettuce seeds (Fig. 5). Because knowledge of the lettuce genome and expressed sequence tag sequences is limited, it is not known how many *DELLA* genes are present in lettuce; however, most of the genes that are expressed in imbibed lettuce seeds are thought to have already been isolated. In contrast to *Arabidopsis* seeds, the expression of the two *DELLA* genes was not down-regulated after FR/R treatment (Fig. 6A, B). These results were consistent with those in lettuce seeds that showed that GA responsiveness was unaffected by R light treatment (Fig. 6C). The major limitation of our study was that the DELLA protein expression levels were not examined. DELLA proteins are also regulated by changes in protein stability and turnover, and DELLA proteins are rapidly degraded by the ubiquitin-proteasome system when plants are treated with GA (Ueguchi-Tanaka *et al*., 2007). Hence, future studies should examine both the transcript levels and translated products of *LsDELLA* genes.

Regardless, of all of the GA metabolism genes, the expression of *LsGA3ox1* and *LsGA3ox2* is most potently regulated by phytochrome in the hypocotyl end of imbibed lettuce seeds. This supports the view that lettuce seed germination is regulated mainly via the control of endogenous levels of physiologically active GA, rather than the control of GA responsiveness. Therefore, it is suggested that GA signalling is regulated by the control of the endogenous GA content and/or the control of GA responsiveness, which may vary with the plant species or cultivar.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Degenerate primer sets used in cDNA cloning.

Table S2. Specific primer sets used in construction of plasmids for functional analysis.

Table S3. Specific primer sets used in the QRT-PCR.

Table S4. GC-MS analysis of the products converted by GST-LsCPS and GST-LsKS.

Table S5. GC-MS analysis of the methyl ester derivatives of products by recombinant LsKO1 and 2 and LsKAO expressed in yeast.

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