Phytochrome- and Gibberellin-Mediated Regulation of Abscisic Acid Metabolism during Germination of Photoblastic Lettuce Seeds[1][OA]

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Germination of lettuce (*Lactuca sativa*) 'Grand Rapids' seeds is regulated by phytochrome. The action of phytochrome includes alterations in the levels of gibberellin (GA) and abscisic acid (ABA). To determine the molecular mechanism of phytochrome regulation of ABA metabolism, we isolated four lettuce cDNAs encoding 9-cis-epoxycarotenoid dioxygenase (biosynthesis; *LsNCED1–LsNCED4*) and four cDNAs for ABA 8′-hydroxylase (catabolism; *LsABA8ox1–LsABA8ox4*). Measurements of ABA and its catabolites showed that a decrease in ABA level coincided with a slight increase in the level of the ABA catabolite phaseic acid after red light treatment. Quantitative reverse transcription-polymerase chain reaction analysis indicated that ABA levels are controlled by phytochrome through down-regulation of *LsNCED2* and *LsNCED4* expression and up-regulation of *LsABA8ox4* expression in lettuce seeds. Furthermore, the expression levels of *LsNCED4* decreased after GA_1 treatment, whereas the levels of expression of the other two genes were unaffected. The *LsNCED4* expression was also down-regulated by red light in lettuce seeds in which GA biosynthesis was suppressed by AMO-1618, a specific GA biosynthesis inhibitor. These results indicate that phytochrome regulation of ABA metabolism is mediated by both GA-dependent and -independent mechanisms. Spatial analysis showed that after red light treatment, the ABA decrease on the hypocotyl side was greater than that on the cotyledon side of lettuce seeds. Moreover, phytochrome-regulated expression of ABA and GA biosynthesis genes was observed on the hypocotyl side, rather than the cotyledon side, suggesting that this regulation occurs near the photo-perceptive site.

Plants undergo correct morphogenesis by responding to light and adapt to various forms of light. In addition to imbibition, some seeds require light to germinate and are called photoblastic seeds. Photoblastic seeds germinate after irradiation with red light (R; wavelength approximately 600–700 nm), and this effect of R is cancelled by successive irradiation with far-red light (FR; wavelength approximately 700–750 nm). The effects of R and FR are reversible. This type of germination, called photogermination, was discovered by experiments using lettuce (*Lactuca sativa*) 'Grand Rapids' seeds (Borthwick et al., 1952). The energy from wavelengths of R is mainly required for photosynthesis. This strategy allows survival of small seeds in which storage substances are low, such as the seeds of lettuce, tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), and Arabidopsis (*Arabidopsis thaliana*). The typical reversible photoreaction of lettuce seeds allowed Butler et al. (1959) to detect the pigment that acts as the R/FR receptor in extracts from etiolated cotyledons of *Brassica rapa* and etiolated shoots of *Zea mays* using differential spectrophotometry, and this photoreceptor was named phytochrome.

Many studies to elucidate the mechanisms of photoblastic lettuce seed germination have been carried out for over a half century, including biochemical, physical,

and physiological studies, etc. (see many references that have been published). For example, the growth potential induced by R was measured by changing the osmotic pressure using mannitol (Takeba and Matsubara, 1979), and the induced growth potential was found to be due to degradation of storage protein (Takeba and Matsubara, 1977) and increased Glu and Gln levels in the hypocotyls of lettuce seeds (Takeba, 1980a, 1980b). The hypocotyl was found to be the most sensitive site in response to R in the lettuce seeds (Inoue and Nagashima, 1991). The role of the phytohormones GA and abscisic acid (ABA) on regulation of germination of the lettuce seeds is important. Application of bioactive GA mimics the effect of R (Kahn and Goss, 1957; Ikuma and Thimann, 1960), whereas application of ABA inhibits germination induced by R or application of bioactive GA (Kahn, 1968; Sankhla and Sankhla, 1968). Endogenous levels of the bioactive GA GA_1 are up-regulated by phytochrome (Toyomasu et al., 1993), and those of ABA are down-regulated by phytochrome or application of GA_1 in lettuce seeds (Toyomasu et al., 1994). These results suggest that control of the balance between endogenous levels of these two phytohormones is essential for regulation of germination of lettuce seeds by light. Furthermore, endogenous levels of GA_1 are up-regulated by phytochrome through marked up-regulation of a GA 3-oxidase (Fig. 1) gene (*Ls3h1*, which is renamed *LsGA3ox1* in this article) for the last step in GA_1 biosynthesis (Toyomasu et al., 1998) and slight down-regulation of a GA 2-oxidase (Fig. 1) gene (*LsGA2ox2*) for inactivation of GA_1 (Nakaminami et al., 2003). However, the molecular mechanisms of ABA metabolism in lettuce seeds have not been addressed.

ABA is a sesquiterpenoid produced from C-40 carotenoids in plants (Milborrow and Lee, 1998; Hirai et al., 2000; Kasahara et al., 2004). The first committed step in ABA biosynthesis in plants is the oxidative cleavage of 9-cis-epoxycarotenoid to xanthoxin. The oxidative cleavage of cis-epoxycarotenoids catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED; Fig. 1) is the key regulatory step of ABA biosynthesis in higher plants (Nambara and Marion-Poll, 2005). ABA catabolism involves two pathways: hydroxylation and conjugation with Glc (Nambara and Marion-Poll, 2005). Hydroxylation pathways occur by irreversible hydroxylation, mainly at the 8′ position (Cutler and
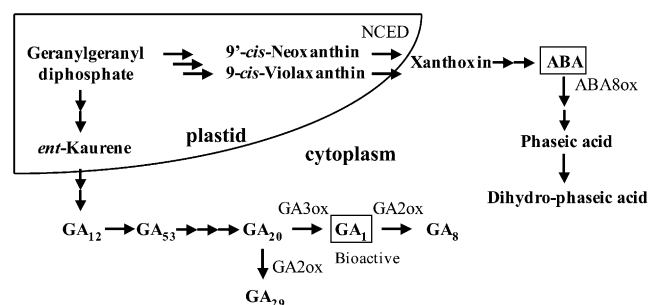

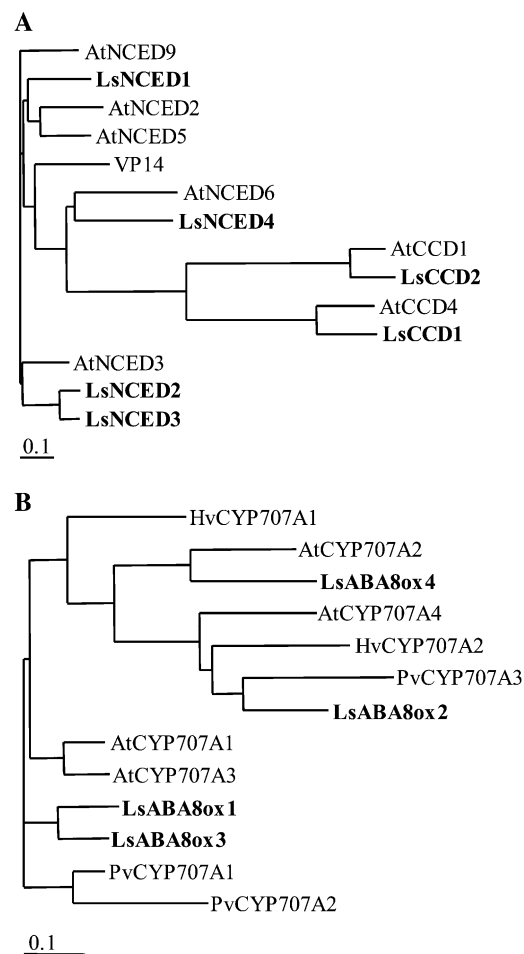**Figure 1.** Metabolism of ABA and GA_1 in lettuce seeds.

Figure 2. Phylogenetic relationships among ABA metabolic enzymes. A, Phylogenetic tree of LsNCED and other plant NCEDs. Analyses were performed using the ClustalW program (http://clustalw.ddbj.nig.ac.jp/top-j.html). The accession numbers are as follows: AtCCD1, NM116217; AtNCED2, NM117945; AtNCED3, NM112304; AtCCD4, NM118036; AtNCED5, NM102749; AtNCED6, NM113327; AtNCED9, NM106486 (Arabidopsis); VP14, U95953 (*Zea mays*); and LsCCD1, LsCCD2, LsNCED1, LsNCED2, LsNCED3, and LsNCED4 (lettuce). B, Phylogenetic tree of LsABA8ox and other plant CYP707As. The accession numbers are as follows: AtCYP707A1, NM118643; AtCYP707A2, NM128466; AtCYP707A3, NM180805; AtCYP707A4, NM112814 (Arabidopsis); HvCYP707A1, DQ145932; HvCYP707A2, DQ145933 (*Hordeum vulgare*); PvCYP707A1, DQ352541; PvCYP707A2, DQ352542; PvCYP707A3, DQ352543 (*Phaseolus vulgaris*); and LsABA8ox1, LsABA8ox2, LsABA8ox3, and LsABA8ox4 (lettuce).

Krochko, 1999; Zhou et al., 2004). The 8′-hydroxyl ABA is spontaneously isomerized to phaseic acid (PA) and reduced to dihydrophaseic acid (DPA) or *epi*-DPA. 8′-Hydroxylation of ABA is catalyzed by ABA 8′-hydroxylase (ABA8ox; Fig. 1), which belongs to the P450 monooxygenase family (Nambara and Marion-Poll, 2005). We focused on NCED and ABA8ox as enzymes responsible for ABA biosynthesis and catabolism, respectively, to investigate ABA metabolism in lettuce seeds. The cDNAs encoding NCED and ABA8ox have been isolated from several plant species, including the

model plant Arabidopsis (Schwartz et al., 1997; Tan et al., 1997; Neill et al., 1998; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Thompson et al., 2000; Iuchi et al., 2001; Kushiro et al., 2004; Saito et al., 2004; Chono et al., 2006; Millar et al., 2006; Yang and Choi, 2006; Yang and Zeevaart, 2006; Saika et al., 2007). Recently, it was reported that ABA levels are controlled by phytochrome through NCED and ABA8ox gene expression in Arabidopsis seeds and that germination is also regulated by phytochrome (Seo et al., 2006).

To elucidate the molecular mechanism regulating ABA levels, we isolated cDNAs encoding NCED and ABA8ox from lettuce and investigated the control of ABA levels and ABA metabolism gene expression by phytochrome. We also performed spatial analysis of endogenous ABA and its metabolic transcripts to compare the ABA reduction site with the photoperceptive site (Inoue and Nagashima, 1991) in lettuce seeds. We discuss ABA metabolism regulated by exogenously applied or endogenous bioactive GA.

RESULTS

Cloning of cDNAs Encoding NCED in Lettuce

One set of degenerate primers (NCED-F and NCED-R) were designed on the basis of conserved amino acid sequences of plant NCED homologs, as described in "Materials and Methods." cDNA fragments of the expected size of approximately 550 bp were amplified from the templates derived from seeds imbibed in the dark and from drought-treated leaves by reverse transcription (RT)-PCR with these degenerate primers. Sequence analysis of the subcloned fragments according to their sequence similarity with other plant NCEDs and carotenoid cleavage dioxygenase (CCD) indicated that four different NCED-like fragments and two CCD-like fragments 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. Six fragments were named *LsNCED1, NCED2, NCED3, NCED4, CCD1,* and *CCD2* (GenBank accession nos. AB120107, AB120108, AB120109, AB120110, AB120111, and AB120112, respectively; Fig. 2A). The predicted coding regions of *LsNCED1, NCED2, NCED3,* and *NCED4* were 1,758,

1,815, 1,752, and 1,737 bp, encoding 585, 604, 583, and 578 amino acid residues, respectively. Those of *LsCCD1* and *CCD2* were 1,779 and 1,626 bp, encoding 592 and 541 amino acid residues, respectively. We could not find a lettuce *NCED* homolog in the database except for these six genes. Identities of amino acid sequences of six clones to the NCED in Arabidopsis (Iuchi et al., 2001) were as follows: LsNCED1/AtNCED5, 68%; LsNCED2/AtNCED3, 70%; LsNCED3/AtNCED3, 72%; LsNCED4/AtNCED6, 57%; LsCCD1/AtCCD4, 67%; and LsCCD2/AtCCD1, 80%. The degrees of identity of each LsNCED with other LsNCEDs and LsCCDs were approximately 50% to 80% and 30%, respectively. Genomic DNA fragments corresponding to these genes were isolated from lettuce and then sequenced. *LsCCD2* included 13 introns, similar to *AtCCD1*, while *LsCCD1* and *LsNCEDs* had no introns, similar to *AtCCD4* and *AtNCEDs* (data not shown), suggesting that these lettuce genes closely resemble the *CCD* and *NCED* genes in Arabidopsis, not only in terms of the sequences but also the gene structures.

We examined whether the products of the four *LsNCED* genes are involved in ABA biosynthesis, while those of the two *LsCCD* genes are not. We confirmed that endogenous levels of ABA are accumulated in transgenic ABA-deficient Arabidopsis *nced3* mutant T5004 (Iuchi et al., 2001) by overexpressing *LsNCED* genes. Transgenic T5004 lines in which each full-length cDNA was constitutively expressed under the control of the cauliflower mosaic virus 35S promoter were prepared, and homozygous T_3 seeds 24 h after the start of imbibition were used to measure ABA content (Fig. 3). The endogenous ABA levels of the imbibed seeds of overexpressed lines of *LsNCED1, NCED2, NCED3,* and *NCED4* were more abundant than those of T5004 seeds. On the other hand, ABA contents in *LsCCD1* and *LsCCD2* overexpressors were the same as those in the parent T5004. Therefore, overexpression of four *LsNCEDs*, but not *LsCCDs*, led to in vivo accumulation of ABA, indicating that these genes encode NCEDs responsible for ABA biosynthesis.

Cloning of cDNAs Encoding ABA8ox in Lettuce

To clone *CYP707A* homologs from lettuce, RT-PCR was performed with five degenerate primers (ABA8ox-F1,

Figure 3. Endogenous levels of ABA in the imbibed seeds of T5004 and transgenic lines. Endogenous levels of ABA in each seed incubated for 24 h under white light were measured by LC-MS/MS. Results are presented as means with SES from at least three independent seed batches. Lowercase letters indicate significant differences in a parameter at $P < 0.05$ (Tukey's honest significant difference test).
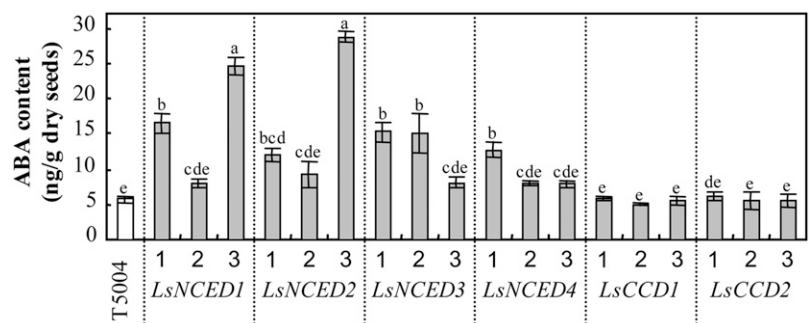
ABA8ox-F2, ABA8ox-R1, ABA8ox-R2, and ABA8ox-R3) designed on the basis of conserved amino acid sequences of *CYP707A* genes in Arabidopsis (Kushiro et al., 2004; Saito et al., 2004). Templates used here were cDNAs derived from R-treated seeds and drought-treated leaves. Three bands of approximately 740 bp (ABA8ox-F1 and ABA8ox-R1) from dehydrated leaves, 790 bp (ABA8ox-F1 and ABA8ox-R2) from R-treated seeds, and 980 bp (ABA8ox-F2 and ABA8ox-R3) from drought-treated leaves were amplified. The results of nucleotide sequence analyses indicated that the PCR products contained four different fragments. These four cDNAs were named *LsABA8ox1*, *LsABA8ox2*, *LsABA8ox3*, and *LsABA8ox4* (GenBank accession nos. AB235917, AB235918, AB235919, and AB235920, respectively; Fig. 2B). The predicted coding regions of *LsABA8ox1*, *ABA8ox2*, *ABA8ox3*, and *ABA8ox4* are 1,495, 1,455, 1,404, and 1,482 bp, encoding 464, 484, 467, and 493 amino acid residues, respectively. The deduced amino acid sequences of *LsABA8ox* showed 75% (LsABA8ox1/CYP707A1), 69% (LsABA8ox2/CYP707A4), 78% (LsABA8ox3/CYP707A1), and 67% (LsABA8ox4/CYP707A2) identity to CYP707A in Arabidopsis.

To confirm that these four cDNAs encode ABA8ox, we carried out in vitro functional analysis. Each cDNA was expressed in yeast harboring an Arabidopsis P450 reductase to yield the recombinant protein (Pompon et al., 1996). PA was identified as a major product in each reaction mixture by liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis (Table I), indicating that each recombinant LsABA8ox catalyzed the 8'-hydroxylation of ABA. These results strongly suggested that *LsABA8ox1* to *4* encode ABA8ox.

Measurement of ABA and Its Catabolites in Germinating Lettuce Seeds

Endogenous levels of ABA decrease after R treatment during germination of lettuce seeds (Toyomasu et al., 1994). Here, we quantified ABA and its catabolites in FR-, FR/R-, and FR/R/FR-treated seeds, prepared as described in "Materials and Methods." FR treatment was used as a control to suppress germination completely. FR/R/FR treatment was conducted to confirm photoreversibility of gene expression, which was expected if these genes are controlled by phytochrome. Radicle emergence was observed from 10 to 12 h after

FR/R treatment under our experimental conditions (Fig. 4A). The endogenous levels of ABA decreased markedly after FR/R treatment, in contrast to the FR control (Fig. 4B). The level of PA was lower than that of ABA in the imbibed seeds, and increased slightly 3 to 9 h after FR/R treatment, compared to the FR control. The effect of R was cancelled out by successive FR treatment (see FR/R/FR). The levels of DPA were lower than those of ABA and were unaffected by light treatment. A peak of *epi*-DPA was not detected in seeds or medium (data not shown). The levels of ABA, PA, and DPA in each culture medium were also measured and were shown to be unaffected by light treatment, suggesting that these compounds in medium had diffused from the seed coats (Inoue, 1990). These results indicated that ABA reduction by R treatment in the seeds was due to metabolism and not to exudation, and catabolic intermediates, such as PA, were quickly catabolized in lettuce seeds.

Gene Expression during Germination

To investigate the regulatory mechanism of endogenous ABA, we performed expression analysis of *LsNCED* and *LsABA8ox* during germination by real-time quantitative RT-PCR (QRT-PCR). In a previous study, we showed marked accumulation of the transcripts of *LsGA3ox1*, encoding GA 3-oxidase, after FR/R treatment (Toyomasu et al., 1998). The *LsGA3ox1* transcript was also quantified as a positive control (Fig. 5A). *LsNCED2* and *LsNCED4* transcripts were predominant in the imbibed lettuce seeds. Transcript levels of *LsNCED1* and *LsNCED3* were much lower than those of *LsNCED2* and *LsNCED4* and were negligible in the imbibed seeds (data not shown). *LsNCED2* expression levels in FR seeds increased slightly 3 h after treatment. In contrast, transcript levels of *LsNCED2* in FR/R seeds decreased 3 h after treatment and continued to decrease gradually until 9 h. A slight increment in *LsNCED2* transcripts in FR seeds was not due to FR treatment, because the expression pattern in the FR seeds was almost the same as that of the imbibed seeds under conditions of continuous darkness (data not shown). The expression levels of *LsNCED4* in the FR/R seeds decreased more drastically than those in the FR seeds 3 h after treatment and continued to decrease gradually until 9 h. On the other hand, *LsABA8ox1* and *LsABA8ox4* transcripts were abundant during seed germination

Table I. *Identification of the reaction product from ABA incubated with microsomes of yeast expressing fusion proteins derived from LsABA8ox*

Clone	Identified Metabolite	Retention Time on LC	Principal Ions/Relative Abundance
		min	*% of base peak*
LsABA8ox1	PA	14.6	279 (M^+, 12), 235 (6), 205 (46), 187 (20), 168 (29), 139 (100)
LsABA8ox2	PA	14.6	279 (M^+, 12), 235 (6), 205 (46), 187 (20), 168 (29), 139 (100)
LsABA8ox3	PA	14.6	279 (M^+, 12), 235 (6), 205 (46), 187 (20), 168 (29), 139 (100)
LsABA8ox4	PA	14.6	279 (M^+, 12), 235 (6), 205 (46), 187 (20), 168 (29), 139 (100)
	Authentic PA	14.6	279 (M^+, 12), 235 (6), 205 (46), 187 (20), 168 (29), 139 (100)

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among the *LsABA8ox* genes (Fig. 5A), and *LsABA8ox2* and *LsABA8ox3* transcript levels were negligible because of their much lower expression levels (data not shown). *LsABA8ox4* transcript levels in FR/R seeds were higher than those in FR seeds at 3 h after treatment, while *LsABA8ox1* transcripts were not markedly affected by light treatment. Successive FR treatment cancelled the effects of R on controlling the levels of *LsNCED2*, *LsNCED4*, and *LsABA8ox4* expression (Fig. 5B). These results suggest that *LsNCED2* and *LsNCED4* were downregulated and *LsABA8ox4* was up-regulated by phytochrome in the imbibed lettuce seeds.

As reported previously, endogenous levels of ABA decreased after application of bioactive GA, which induced lettuce seed germination (Toyomasu et al., 1994). To examine whether *LsNCED* and *LsABA8ox* are regulated by exogenously applied GA, we performed QRT-PCR on GA₁-treated seeds, prepared as described in "Materials and Methods." The expression levels of *LsNCED4* decreased in the GA₁-treated seeds and were delayed relative to FR/R seeds, while there were no marked effects on the expression of other genes in comparison to the FR control (Fig. 5A). These patterns of decrease in the *LsNCED4* transcript reflect the frequency of germination (Fig. 4A). The results strongly suggest that down-regulation of *LsNCED4* was responsible for the ABA decrease after GA₁ treatment. Therefore, we presume that *LsNCED4* expression is down-regulated by endogenous GA₁ accumulated in FR/R seeds. However, we could not entirely eliminate the possibility that *LsNCED4* expression is also down-regulated by phytochrome. To examine whether the expression of *LsNCED4* is also regulated by phytochrome, we carried out QRT-PCR using seeds in which GA biosynthesis was suppressed by treatment with GA biosynthetic inhibitor. A recent study showed that triazole-type GA biosynthetic inhibitors, such as uniconazole-P, inhibit ABA8ox activity (Saito et al., 2006) as well as *ent*-kaurene oxidase activity (Izumi et al., 1985). Both of these enzymes belong to the P450 monooxygenase family. Therefore, we used AMO-1618, which suppresses the early step (*ent*-copalyl diphosphate synthase, a member of the diterpene cyclase family) of GA biosynthesis (Kawaide et al., 1997). When we used 50 mg of seeds in the same volume of buffer and petri dish as in the above experiments, inhibition of GA biosynthesis by AMO-1618 suppressed germination up to approximately 5% (Fig. 6A). AMO-1618 treatment did not suppress down-regulation of *LsNCED4* after FR/R (Fig. 6B), although the expression pattern in FR seeds using 50 mg of seeds was different from that using 500 mg of seeds (Figs. 5A and 6B). These results suggest that expression of *LsNCED4* is down-regulated by phytochrome as well as GA.

Spatial Analysis of Endogenous ABA and Transcripts of *LsNCED* and *LsABA8ox*

To examine the site of the ABA decrease in germinating seeds, we measured endogenous levels of ABA

in seeds divided into two parts after freezing defined as the cotyledon side or the hypocotyl side. The cotyledon side was composed of the cotyledon, fruit wall, seed coat, and endosperm, while the hypocotyl side included the hypocotyl, root apex, shoot apex, part of the cotyledon, fruit wall, seed coat, and endosperm (Fig. 7A). Endogenous ABA levels in the imbibed seeds in the dark, just before light treatment, were higher on the hypocotyl side than on the cotyledon side (Fig. 7B; 0 h on the x axis). The ABA levels on both sides of the FR/R seeds decreased more markedly than those in the nongerminating FR seeds (Fig. 7B; compare white symbols and gray symbols). For example,
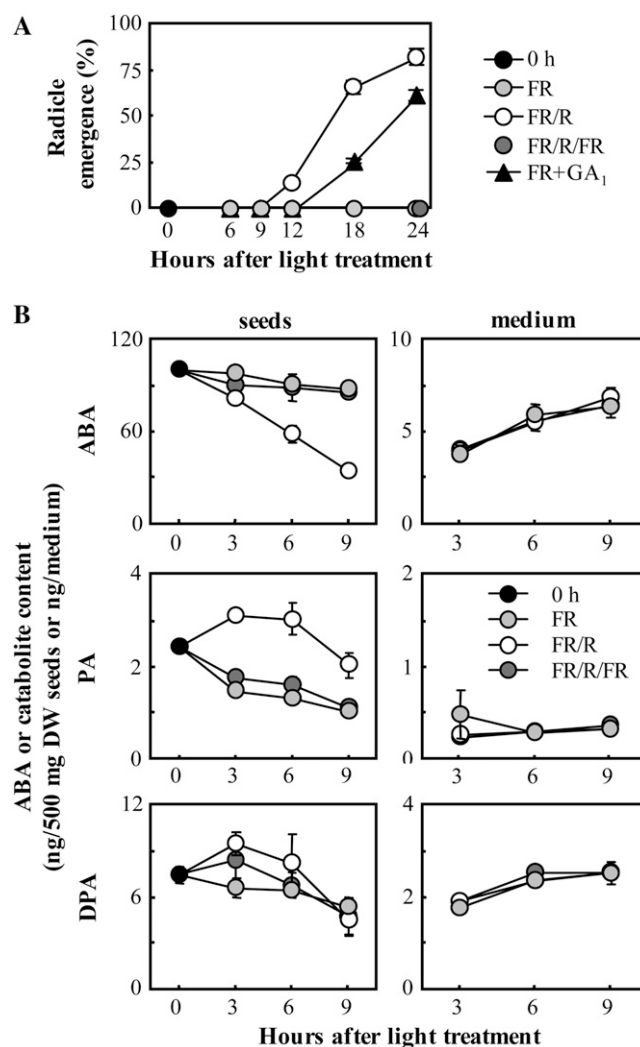

Figure 4. Quantification of endogenous ABA, PA, and DPA levels during incubation under different light conditions. A, Time course of germination of lettuce seeds under different conditions. 0 h indicates seeds imbibed for 3 h in the dark (just before light treatment). FR+GA₁ indicates seeds treated with FR and 1 mM GA₁. Experiments were performed in triplicate, and averages are shown with SEs. B, The endogenous levels of ABA, PA, and DPA were analyzed by LC-MS/MS. DW, Dry weight. Experiments were performed in duplicate and averages are shown with SEs.
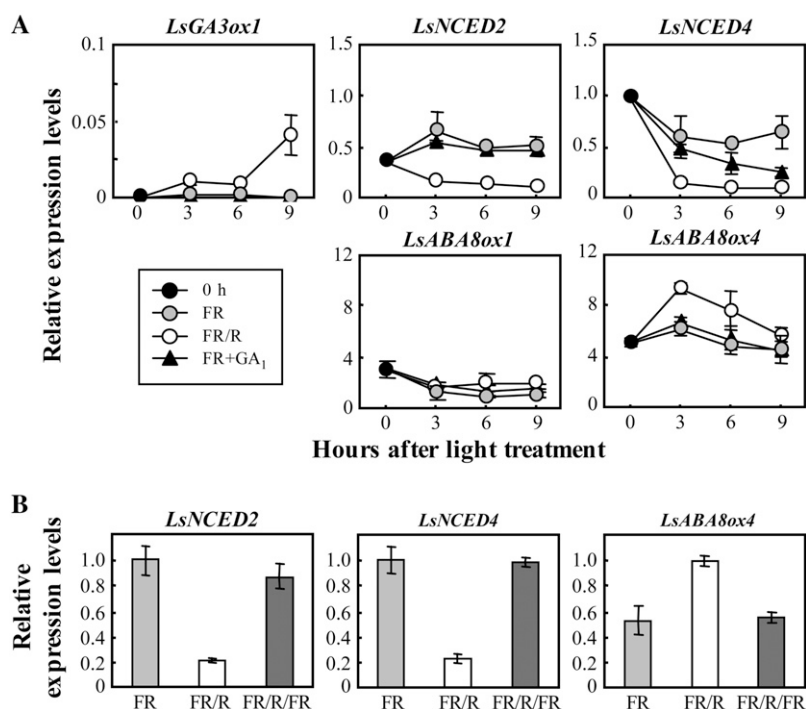
Figure 5. Changes in the transcript levels of *LsNCED* and *LsABA8ox* during incubation under different conditions. A, Expression levels of *LsNCED* and *LsABA8ox* after light and GA$_1$ treatment. The expression levels of these genes were analyzed by QRT-PCR as described in the text. 0 h indicates seeds imbibed for 3 h in the dark (just before light treatment). FR+GA$_1$ indicates seeds treated with FR and 1 mM GA$_1$. The results were normalized to the expression of 18S rRNA (internal control), and the expression levels of all genes examined are given relative to a reference value, the transcript level of *LsNCED4* at 0 h, set to 1. Two independent experiments were performed, and averages are shown with SEs. B, Expression levels of *LsNCED* and *LsABA8ox* under photoreversible conditions 3 h after light treatment. The results were normalized to the expression of 18S rRNA, and then the highest value of each gene was set equal to 1. Two independent experiments were performed, and averages are shown with SEs.

ABA content in FR/R seeds minus those in FR seeds was −36 pg/seed on the hypocotyl side and −25 pg/seed on the cotyledon side 9 h after light treatment. The decrease by R of ABA content on the hypocotyl side was 1.4-fold greater than that on the cotyledon side.

To assess the site of *LsNCED* and *LsABA8ox* expression in seed germination, in situ hybridization was performed, but no positive signals were detected because of the low expression levels. We then carried out expression analysis of *LsNCED* and *LsABA8ox* on the divided seeds described above by QRT-PCR. We also performed expression analysis of GA metabolic enzyme genes, *LsGA3ox1* and *LsGA2ox2*, to compare the sites of GA$_1$ accumulation and *LsNCED4* expression, which may be down-regulated by endogenous GA$_1$ in germinating seeds. The results of expression analysis of the GA and ABA metabolic enzyme genes are presented in Figure 7C. Transcripts of *LsGA3ox1* and *LsGA2ox2* were detected mainly on the hypocotyl side rather than the cotyledon side. The transcript levels of *LsGA3ox1* and *LsGA2ox2* on the hypocotyl side of the FR/R seeds were much higher and lower, respectively, than those of the FR seeds. Similarly, transcript levels of *AtGA3ox1* and *AtGA2ox2* were detected in the cortex and endodermis in the hypocotyl of Arabidopsis (Yamaguchi et al., 2001; Yamauchi et al., 2007). These results suggest that endogenous GA$_1$ accumulated much more on the hypocotyl side than the cotyledon side in lettuce seeds. *LsNCED2* transcripts were also detected predominantly on the hypocotyl side and decreased markedly after FR/R treatment. *LsNCED4* transcripts were detected equally on both sides of the

seed just before light treatment (0 h). The expression levels of *LsNCED4* on the hypocotyl side decreased markedly after FR/R treatment, whereas expression on the cotyledon side showed almost equivalent decreases after FR and after FR/R treatment. The almost equivalent expression levels of *LsNCED4* on the hypocotyl side of the FR seeds during incubation suggested that the reduction of *LsNCED4* levels in the whole FR seeds (Fig. 5A) was due to their reduction on the cotyledon side. In contrast, *LsABA8ox4* transcripts accumulated equally on both sides and increased after FR/R irradiation. These results indicated that *LsNCED2* and *LsNCED4* are mainly associated with a decrease in ABA on the hypocotyl side.

DISCUSSION

Lettuce seeds are excellent plant materials in which to investigate photogermination, and they have been used in many studies. The size (approximately 5 mm) and structure (straight embryo in a seed coat) of lettuce seeds are suitable for localization analysis. Inoue and Nagashima (1991) found the photoperceptive site in lettuce seeds by partial irradiation of the seeds with R through slit irradiation. The levels of Glu and Gln were also found to be increased in hypocotyls after R and GA (Takeba, 1980a, 1980b, 1980c, 1980d). Arabidopsis seeds, which are also photoblastic, are also suitable materials for genetic analysis and have been used in many studies of the function of various target genes for regulation of plant development since the 1990s. Using Arabidopsis seeds and their mutants or trans-
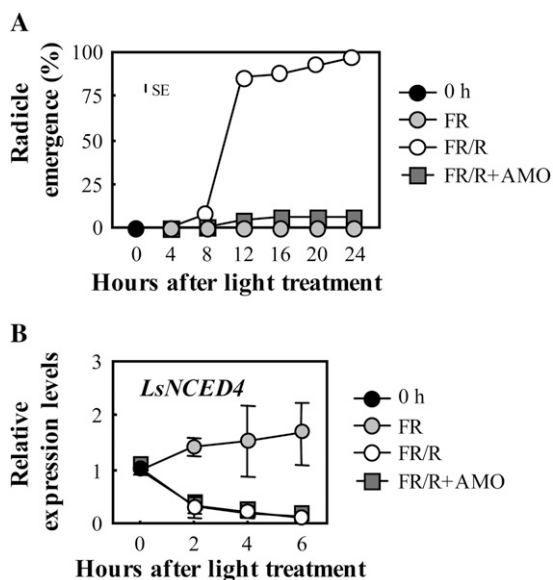
Figure 6. Changes in transcript levels of *LsNCED4* during incubation with GA biosynthetic inhibitor. A, Time course of germination of lettuce seeds under different conditions. 0 h indicates seeds imbibed for 3 h in the dark (just before light treatment). Experiments were performed in triplicate and averages are shown with SES. The vertical bars represent the maximum size of the SE bar within the figure. B, Expression levels of *LsNCED4* in the seeds incubated in medium containing 50 mM AMO-1618. The expression levels of these genes were analyzed by QRT-PCR as described in the text. The results were normalized to the expression of 18S rRNA (internal control), and then the expression level at 0 h was set to 1. Two independent experiments were performed, and averages are shown with SES.

genic plants, characterization of the phytochrome family (Shinomura et al., 1996) and metabolic enzyme genes of GAs and ABA (Yamaguchi et al., 1998, 2001; Ogawa et al., 2003; Seo et al., 2006; Oh et al., 2007) provided important information on the mechanism of photo-germination.

Our results indicated that down-regulation of *LsNCED2* and *LsNCED4* expression and up-regulation of *LsABA8ox4* expression are involved in the decrease in ABA in lettuce seeds (Fig. 8). However, regulation of ABA metabolism by phytochrome in lettuce seeds was different from that in Arabidopsis seeds in terms of the involvement of GA. GA-independent signals appeared to contribute more prominently to phytochrome regulation of ABA levels in lettuce seeds than in Arabidopsis seeds. In lettuce, *LsNCED2* and *LsABA8ox4* were regulated by GA-independent signals, whereas *LsNCED4* was regulated by both GA-dependent and GA-independent signals (Figs. 5A and 6B). This is in contrast to phytochrome regulation in Arabidopsis, in which *AtNCED6* and *AtCYP707A2* are regulated by both GA-dependent and GA-independent signals (Seo et al., 2006; Oh et al., 2007). These results indicate that the molecular mechanisms of regulation of ABA levels by phytochrome are partly different and that the detailed mechanisms vary according to plant species.

In previous studies using Arabidopsis seeds, in situ hybridization and reporter gene analysis were carried out to estimate localization of GAs and ABA (Yamaguchi et al., 2001; Okamoto et al., 2006; Seo et al., 2006; Yamauchi et al., 2007). However, these methods were not suitable for measurement of the precise content of the target mRNA, and, moreover, localization of the phytohormones could not be addressed because of the small size and curved embryos in Arabidopsis seeds. In this study, we measured ABA levels in dissected hypocotyl and cotyledon parts of lettuce by LC-MS/ MS. This analysis demonstrated that the photoperceptive hypocotyl side showed more abundant reduction in ABA levels than the cotyledon parts (Fig. 7B). This is the first report, to our knowledge, of spatial information of the decrease in ABA by R in plant seeds. Consistent with these changes in ABA level, QRT-PCR expression analysis in dissected seeds showed that phytochrome regulation of *LsNCED2* and *LsNCED4* expression mainly occurred in the hypocotyl parts. We could not measure GA levels in each part, because the endogenous GA_1 levels were approximately 1/100 those of ABA in lettuce seeds. However, the good agreement of changed spatial contents of ABA and expression patterns of ABA metabolism genes in lettuce seeds suggested that GA levels also increase in hypocotyls through up-regulation of *LsGA3ox1* and down-regulation of *LsGA2ox2* expression. As the hypocotyl was shown to be the most sensitive site in response to R in lettuce seeds (Inoue and Nagashima, 1991), it was suggested that levels of bioactive GA increase and those of ABA decrease near the photoperceptive site in the hypocotyls of lettuce seeds. Previous studies indicated that the growth potential of the hypocotyl in halved lettuce seeds is induced by R (Takeba, 1980d) or GA (Takeba, 1980c), and accumulation of Glu and Gln in the hypocotyls through up-regulation of Gln synthase gene expression by R or GA results in an increment in growth potential (Takeba, 1983, 1984; Sakamoto et al., 1990). These spatial analyses also suggest that biochemical events occurring in the hypocotyl of lettuce seeds are essential for germination in light. Recently, it was shown that phytochrome regulates GA and ABA metabolism genes in Arabidopsis seeds through PIL5, a negative regulator of phytochrome signals (Oh et al., 2007). Future studies should address whether photoperceptive cells are identical to the cells in which the levels of phytohormones change.

We were able to discriminate between universal and species-specific events by comparing the results obtained in lettuce and Arabidopsis. The information obtained by experiments using lettuce provides novel insights into the conserved and diverse mechanisms of photogermination among plant species. Further studies using Arabidopsis and/or lettuce seeds, such as investigation of the events in hypocotyls in seeds, will provide further important information to help us to understand the mechanisms of regulation of seed germination by light.
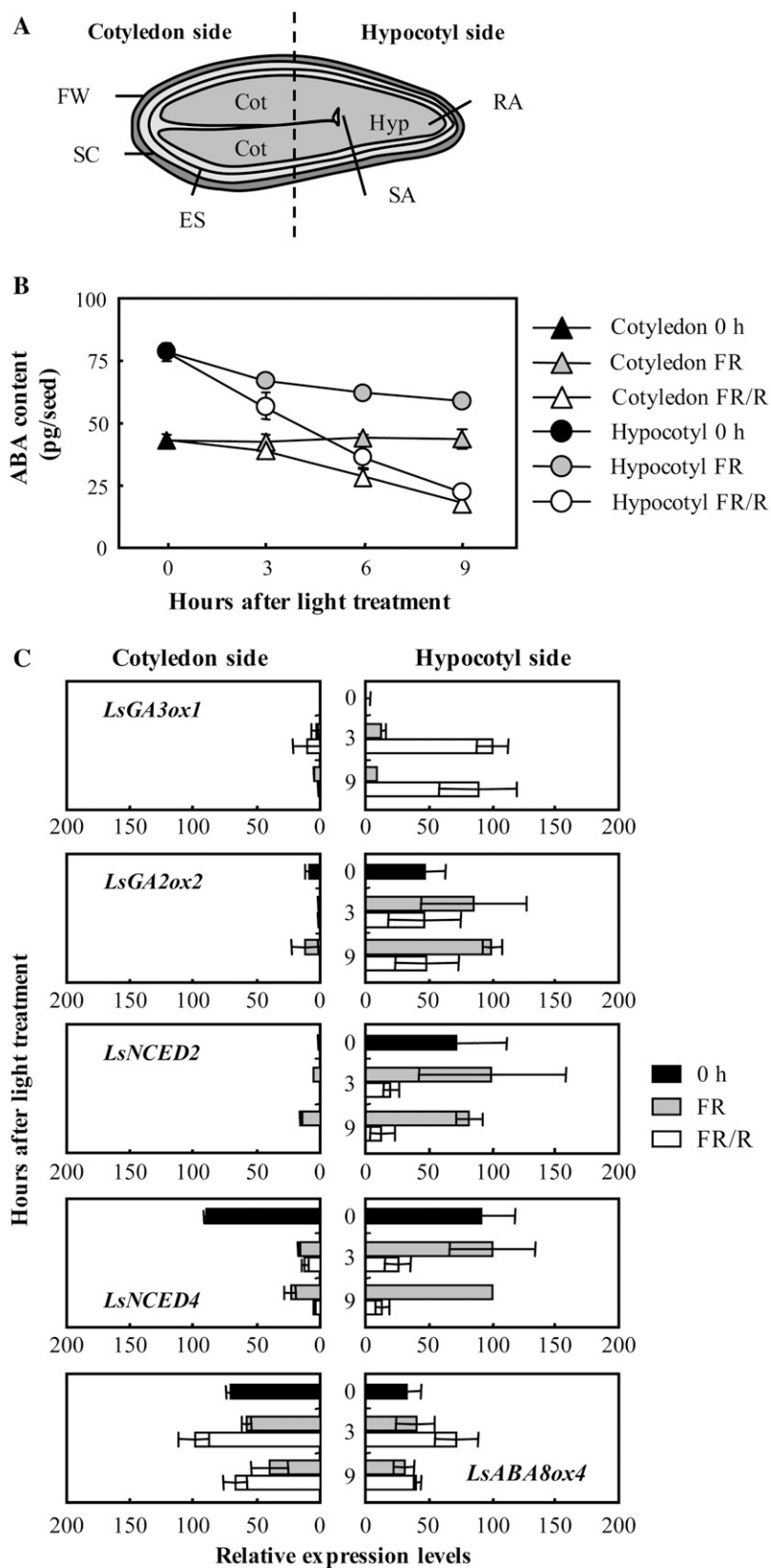
Figure 7. ABA and GA metabolism on the cotyledon side and the hypocotyl side of lettuce seeds. A, Frozen seeds were divided into two parts: cotyledon side, including the cotyledons (Cot), fruit wall (FW), seed coat (SC), and endosperm (ES); and the hypocotyl side, including the hypocotyl (Hyp), root apex (RA), shoot apex (SA), and part of the Cot, FW, SC, and ES. B, Quantification of endogenous ABA levels in the cotyledon side and the hypocotyl side by LC-MS/MS. 0 h indicates seeds imbibed for 3 h in the dark (just before light treatment). Experiments were performed in triplicate and averages are shown with SES. C, Changes in transcript levels of GA and ABA metabolism genes by QRT-PCR. The results were normalized to the expression of 18S rRNA (internal control), and then the highest value was set to 100. Two independent experiments were performed, and averages are shown with SES.
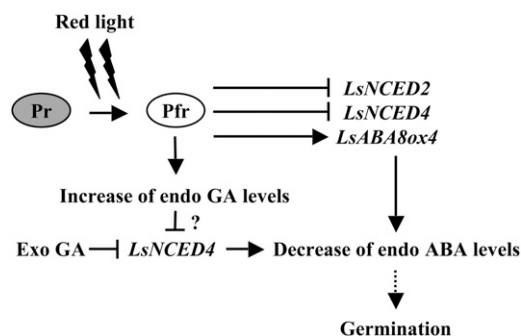
Figure 8. Model of the regulatory mechanism of ABA levels on the germination of photoblastic lettuce seeds. Evidence for this model is discussed in the text. Arrows indicate activation, and T bars indicate negative regulation. Question mark indicates suggested relationships.

MATERIALS AND METHODS

Light Sources and Plant Materials

R ($7\ \mathrm{W\,m^{-2}}$) was supplied by light-emitting diodes (MIL-R18; Sanyo Biomedical). FR ($5.5\ \mathrm{W\,m^{-2}}$) was also supplied by light-emitting diodes (MIL-R18) passed through a far-red acrylic filter (Deraglass A900, 2 mm thick; Asahikasei). Lettuce (*Lactuca sativa*) 'Grand Rapids' seeds were obtained by propagation of the same lot of seeds as used in our previous studies (Toyomasu et al., 1998; Nakaminami et al., 2003) obtained from South Pacific Seed in January 2005 and stored at 4°C with silica gel until use. Seeds (500 mg or 50 mg) were incubated according to the method described previously (Nakaminami et al., 2003). Three different light treatments were applied to seeds 3 h after the start of imbibition: irradiation with FR (FR treatment); FR followed by R (FR/R treatment); and FR, R, and FR, successively (FR/R/FR treatment). Each light irradiation lasted 5 min. Seeds imbibed in the dark for 3 h were also harvested (0 h) just before the light treatment. After light treatment, seeds were incubated in the dark at 25°C for 3, 6, and 9 h (500 mg) or 2, 4, and 6 h (50 mg). The seeds were then harvested and frozen in liquid nitrogen. Application of GA_1 (1 mM) to the seeds was carried out as described previously (Nakaminami et al., 2003). Application of AMO-1618 (50 mM) to the seeds was performed at the start of imbibition. For dark incubation, seeds were handled under a dim-green safety light.

Lettuce seeds were sown on vermiculite and grown under continuous white light (6,000 lux) at 25°C for 1 month in a plant growth chamber (Biotron LPH 200; NK Systems). Plants were harvested and placed on filter paper under continuous white light at 25°C for 3 h. The dried leaves were collected and frozen. Total RNA was isolated and used for molecular cloning of ABA metabolic enzyme genes.

Molecular Cloning

The design of the degenerate primers for *NCED* was based on conserved amino acid regions of the NCED of *Zea mays* (Schwartz et al., 1997; Tan et al., 1997), *Solanum lycopersicum* (Thompson et al., 2000), Arabidopsis (*Arabidopsis thaliana*; Iuchi et al., 2001), *Phaseolus vulgaris* (Qin and Zeevaart, 1999), and *Persea americana* (Chermys and Zeevaart, 2000). The sequences of these oligonucleotides were 5′-TNTTYGAYGGNGAYGGNATG-3′ (NCED-F; for-

ward primer, encoding LFDGDGM) and 5′-GTRCTRAARCGNTADTGNCT-3′ (NCED-R; reverse primer, encoding HDFAITE). The design of five degenerate primers for *ABA8ox* was based on conserved amino acid regions of the CYP707A family of Arabidopsis (Kushiro et al., 2004; Saito et al., 2004). The sequences of these oligonucleotides were 5′-GCNYTITTYTTYCAICARGG-3′ (ABA8ox-F1; forward primer, encoding AIFFHQG), 5′-TNGGNTGYCCNT-GYGTNATG-3′ (ABA8ox-F2; forward primer, encoding LGCPCVN), 5′-CAN-GCYTCICKRAAIGTRAA-3′ (ABA8ox-R1; reverse primer, encoding VAERFTF), 5′-ACYTTCCANCCYTTIGGDAT-3′ (ABA8ox-R2; reverse primer, encoding VKWGKPI), and 5′-GGYTTNGGNGCNACYTCRAA-3′ (ABA8ox-R3; reverse primer, encoding PKPAVEF). The template cDNA for PCR was as described previously (Toyomasu et al., 1998) and was also applied to the dehydrated leaves. The reaction mixture (50 μL) contained 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 μM of each primer, and 2.5 units of Expand HF (Roche Diagnostics). The samples were heated to 94°C for 2 min, and then subjected to 40 cycles of 94°C for 1 min, 50°C (NCED) or 45°C (ABA8ox) for 1 min, and 72°C for 1 min, with a final extension of 72°C for 7 min. PCR fragments were cloned with the pGEM-T Easy vector (Promega). DNA sequencing was performed using a Big Dye terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). RACE was performed using a Marathon cDNA amplification kit (CLONTECH) according to the methods described previously (Toyomasu et al., 1998).

Sequence Similarity Search and Alignment of Amino Acid Sequences

Homology searches of the databases were performed with BLAST (http://www.arabidopsis.org/Blast/index.jsp). Alignment of amino acid sequences was carried out using ClustalW, available from the DNA Data Bank of Japan home page (http://clustalw.ddbj.nig.ac.jp/top-j.html).

Functional Analysis of *LsNCED* by Heterologous Expression

Full-length cDNA was amplified by PCR using primers with restriction enzyme sites for *Xho*I at the 5′-end and *Kpn*I at the 3′-end. The sequences of the primers used are shown in Table II. PCR fragments were subcloned into the pGEM-T Easy vector. The plasmids were digested with *Xho*I and *Kpn*I and ligated into the Gateway entry vector pENTR1A (Invitrogen). The inserts were cloned into the pGWB2 vector (provided by Dr. Tsuyoshi Nakagawa, Shimane University) by the LR reaction according to the manufacturer's protocol. The plasmids were introduced into *Agrobacterium tumefaciens* by electroporation. T-DNA was transferred into the *nced3* mutant T5004 (Iuchi et al., 2001) by the floral dipping method (Clough and Bent, 1998). T_1 transgenic seeds were selected on half-strength Murashige and Skoog medium containing 0.8% (w/v) agar, kanamycin and hygromycin (each 25 μg/mL), and 100 μg/mL of chloramphenicol. T_1 seedlings were transferred to soil and grown at 22°C under continuous white light. T_2 plants were grown under the same conditions. Samples of 10 mg of seeds (T5004 and homozygous T_3 transgenic lines) were placed on three filter papers (Filter Papers 3; Whatman) moistened with 2 mL of water in 60-mm tissue culture dishes (Iwaki). The seeds were incubated at 22°C under white light for 24 h and then harvested. Samples were extracted and subjected to LC-MS/MS analysis using 0.2 ng ($\pm$)-2-cis, 4-trans-$ABA\text{-}d_6$ (Icon Services) as an internal standard, as described below in the section "Extraction and Mass Spectrometric Analysis of ABA, PA, and DPA." Measurements of ABA in seeds of T5004 and each transformant line were taken from six and three independent seed batches, respectively.

Table II. *Specific primer sets used in construction of transgenic plants*
Underlined text and underlined, bold text show the *Kpn*I and the *Xho*I site, respectively.

Gene Name	Forward Primer	Reverse Primer
LsCCD1	GGTACCCAGACCACACAATGGATTCTC	CTCGAGACTTAAAACTGATGTTTCACTTAT
LsCCD2	GGTACCCACAGATGGGCACCATGGAG	CTCGAGAATTGATAACACCATTGTATTAAT
LsNCED1	GGTACCACCTCCAGCCCTTCATTCCT	CTCGAGGAAAAGCACATTCATGTTTCTTCA
LsNCED2	GGTACCCGACATTAAAATAGCTTCAATCAC	CTCGAGAGATGAAATTTTACATAGTTATATA
LsNCED3	GGTACCTCCACCAACTTTCAAATTGCTTAC	CTCGAGACTTAATACAACATAAAGTATATT
LsNCED4	GGTACCCACCATTAAAGCTATCACGT	CTCGAGATTGAATTAAGCTTAATTAGCCAA

Table III. *Specific primer sets used in the real-time QRT-PCR*

Gene Name	Forward Primer	Reverse Primer
18SrRNA	GGAGCGATTTGTCTGGTTA	ATCTAAGGGCATCACAGACC
LsGA3ox1	TGTTTGCACTTCCTATCCAACAG	TACGAGCATGTTCGTATGGAGAT
LsGA2ox2	TGGAGGTCCACCATTGATG	CCTATTATCAGTCAACCTGGTCTTGTAG
LsNCED1	AAACCCTACAATCCGACTATTCG	GGCCGCAGCTCTTTGTAAG
LsNCED2	CTTCAGTTTCCTAAACAGTCTGTTGGTA	TGCTTTCAATCCATCTTCAACG
LsNCED3	CAGTCGTCGGAGAAATTCCA	GCCTTTGTGTCTCCGTATGG
LsNCED4	GGACACGGCTCATGGAATC	GCGAGATCACCGTCACGTT
LsABA8ox1	GCTCGTGACCAAATCTCATCTC	TCAATGTGGGAAACCATGTG
LsABA8ox2	TTGGGTTGTCCAAGTATCATGTTAG	CAAGCTTCGGAGATTATCAAGTGAT
LsABA8ox3	CCTGCTAGCAAAGAGAGAATGC	CCCATCCTCGGAGTGATTG
LsABA8ox4	GTGATGATATCGAGTCCTAAAGTAGCA	TTGAGATGCGAATGGTATGG

Functional Expression of Lettuce ABA8ox

Functional analysis of LsABA8ox1 to LsABA8ox4 was performed using the method described by Kushiro et al. (2004). Full-length cDNA was cloned into a yeast (*Saccharomyces cerevisiae*) expression vector, pYeD60 (Pompon et al., 1996). The plasmid was transformed into yeast strain WAT11 (Pompon et al., 1996). Recombinant yeasts were grown in SGI medium for 24 to 36 and then transferred to SLI medium and induced by adding Gal for 12 h. The cell pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.6, and passed through a French press (20,000 psi). Microsomal fractions were suspended in 0.1 M potassium phosphate buffer, pH 7.6. To assay ABA 8′-hydroxylase, (+)-S-ABA was incubated with 2 μg of microsomal protein (in a volume of 100 μL) containing 0.5 mM NADPH, 0.5% (w/v) Triton X-100, and 1 mM ($\pm$)-dithiothreitol at 22°C for 16 h. The reaction was stopped by adding 1 N HCl, extracted with ethyl acetate, and dried by centrifugation under vacuum. The resulting sample mixture was dissolved in water and analyzed by LC-MS/MS. LC-MS/MS analysis was performed using the same method as described in the next section.

Extraction and Mass Spectrometric Analysis of ABA, PA, and DPA

ABA and its metabolites in seed samples were extracted and quantified as described previously (Saika et al., 2007) using 50 ng of ($\pm$)-2-cis, 4-trans-ABA-d_6, 2.5 ng of d_3-PA, d_3-DPA (provided by Dr. Suzanne R. Abrams, National Research Council of Canada), and d_3-*epi*-DPA (provided by Dr. Nobuhiro Hirai, Kyoto University) as internal standards. The culture media were collected from dishes, and then 10 ng of ($\pm$)-2-cis, 4-trans-ABA-d_6, 1 ng of d_3-PA, d_3-DPA, and d_3-*epi*-DPA were added. The culture media were adjusted to pH 2 with 1 N HCl and extracted with ethyl acetate. The dehydrated ethyl acetate fractions were dried by centrifugation under vacuum. Extracts were dissolved in 300 μL of methanol and again dried by centrifugation under vacuum and subjected to LC (ACQUITY UPLC system; Waters)-MS/MS (Q-Tof premier; Micromass). The following fragments were used for quantification: 153 (ABA), 159 (d_6-ABA), 139 (PA), 142 (d_3-PA), 171 (DPA), 174 (d_3-DPA), 171 (*epi*-DPA), and 174 (d_3-*epi*-DPA). For ABA measurement from separated seed, frozen seeds (20 seeds) were separated into two parts on dry ice. Samples were extracted and subjected to LC-MS/MS analysis using 1 ng of ($\pm$)-2-cis, 4-trans-ABA-d_6 as an internal standard, according to the method described previously (Saika et al., 2007).

Real-Time QRT-PCR

Total RNA was extracted from frozen seeds by the hot borate method (Wan and Wilkins, 1994) or on an RNAqueous column with Plant RNA Isolation Aid (Ambion). For expression analysis of divided seeds, samples of 50 mg of frozen seeds (fresh weight) were divided into two parts using a razor on dry ice. Total RNA was extracted from each portion using the RNAqueous column with Plant RNA Isolation Aid. cDNA was synthesized from aliquots of 1 μg total RNA using a QuantiTect Reverse Transcription kit (Qiagen). Real-time QRT-PCR using SYBR Green I was carried out on a sequence detector system (model 7000; Applied Biosystems) or a Thermal Cycler Dice real-time system (TP800; TaKaRa) as described previously (Kushiro et al., 2004). The sequences of the primers used are shown in Table III. The mean values of two or three

replicates were normalized using 18S ribosomal RNA (rRNA) as an internal control.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers LsNCED1: AB120107; LsNCED2: AB120108; LsNCED3: AB120109; LsNCED4: AB120110; LsCCD1: AB120111; LsCCD2: AB120112; LsABA8ox1: AB235917; LsABA8ox2: AB235918; LsABA8ox3: AB235919; LsABA8ox4: AB235920.

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