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RESEARCH PAPER

An *Arabidopsis* mutant able to green after extended dark periods shows decreased transcripts of seed protein genes and altered sensitivity to abscisic acid

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Abstract

An *Arabidopsis* mutant showing an altered ability to green on illumination after extended periods of darkness has been isolated in a screen for *genomes uncoupled* (*gun*) mutants. Following illumination for 24 h, 10-day-old dark-grown mutant seedlings accumulated five times more chlorophyll than wild-type seedlings and this was correlated with differences in plastid morphology observed by transmission electron microscopy. The mutant has been named *greening after extended darkness 1* (*ged1*). Microarray analysis showed much lower amounts of transcripts of genes encoding seed storage proteins, oleosins, and late embryogenesis abundant (LEA) proteins in 7-day-old seedlings of *ged1* compared with the wild type. RNA gel-blot analyses confirmed very low levels of transcripts of seed protein genes in *ged1* seedlings grown for 2–10 d in the dark, and showed higher amounts of transcripts of photosynthesis-related genes in illuminated 10-day-old dark-grown *ged1* seedlings compared with the wild type. Consensus elements similar to abscisic acid (ABA) response elements (ABREs) were detected in the upstream regions of all genes highly affected in *ged1*. Germination of *ged1* seeds was hypersensitive to ABA, although no differences in ABA content were detected in 7-day-old seedlings. This suggests the mutant may have an altered responsiveness to ABA, affecting expression of ABA-responsive genes and plastid development during extended darkness.

Key words: abscisic acid, *Arabidopsis*, greening, photosynthesis genes, seed protein genes.

Introduction

Higher plants assume different growth patterns under dark or light conditions during early development (McNellis and Deng, 1995). Before emerging from the soil, dicotyledonous seedlings follow skotomorphogenic (etiolated) growth, showing elongated hypocotyls with small folded cotyledons and an apical hook (Staub and Deng, 1996). The etiolated seedlings show little or no expression of photosynthesis-related genes and do not contain developed chloroplasts (Chory *et al*., 1996; Staub and Deng, 1996). When the seedlings emerge into the light, the growth is switched to photomorphogenic development that is accompanied by inhibition of hypocotyl elongation, open and expanded cotyledons, lack of an apical hook, developed chloroplasts, and photosynthetic gene expression (Chory *et al*., 1996; Staub and Deng, 1996). Developing seedlings devote their nutritional reserves, including storage proteins and lipids, almost exclusively to hypocotyl extension, and develop machinery for photosynthesis after reaching the light (McNellis and Deng, 1995). Seed storage proteins are degraded during germination to supply amino acids for development (Fujiwara *et al*., 2002), and storage lipids are mobilized to provide an energy source for the seedlings (Penfield *et al*., 2006*b*).

Photomorphogenesis is facilitated through several photoreceptors recognizing different regions of the light

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spectrum, such as phytochromes (red/far-red photoreceptors) (Quail *et al*., 1995), cryptochromes (Cashmore *et al*., 1999) and phototropins (Briggs and Christie, 2002) (blue/UV-A receptors), and unknown photoreceptor(s) for UV-B (Brosché and Strid, 2003). In addition, phytohormones appear to play a role in light-dependent seedling development, especially, in some cases, as downstream effectors of the phytochrome transduction pathway (Wei and Deng, 1996; Nemhauser and Chory, 2002). All major phytohormones appear to interact with the components of light signalling and have been implicated in photomorphogenesis, with cytokinins promoting photomorphogenesis, and auxin, brassinosteroids, and gibberellins showing an opposite action (Chory *et al*., 1994; Nemhauser and Chory, 2002). An abscisic acid (ABA) response, acting in opposition to brassinosteroids and gibberellins, appears to be required for etiolated development (Rohde *et al*., 2000; Nemhauser and Chory, 2002). ABA INSENSITIVE 3 (ABI3) plays a role in plastid and leaf development in dark-grown *Arabidopsis* seedlings (Rohde *et al*., 2000). ABI4 has also been demonstrated to regulate lipid mobilization that is required to fuel seedling establishment (Penfield *et al*., 2006*a*) and to mediate sugar and ABA responsiveness by direct binding to a light-responsive element (Acevedo-Hernández *et al*., 2005). Recently, ABI4 has been proposed to act downstream of GENOMES UNCOUPLED 1 (GUN1) in the plastid-to-nucleus retrograde signalling pathway to repress nuclear genes encoding plastid proteins (Koussevitzky *et al*., 2007).

Inhibitors of plastid translation cause repression of nuclear gene expression when applied early in the development of seedlings, suggesting that plastid-to-nucleus signalling responding to the state of plastid translation is required in early seedling development for the expression of nuclear genes encoding photosynthesis proteins (Oelmüller *et al*., 1986; Gray *et al*., 1995). Expression of nuclear genes, such as *RBCS* (encoding ribulose-1,5-bisphosphate carboxylase small subunit) and *LHCB1* (encoding light-harvesting chlorophyll *a*/*b*-binding protein 1), in *Arabidopsis gun1* mutants is not sensitive to inhibitors of plastid translation such as chloramphenicol and lincomycin, which down-regulate nuclear photosynthesis genes in wild-type seedlings (Susek *et al*., 1993; Gray *et al*., 2003), suggesting that GUN1 may be involved in the plastid protein synthesis-responsive signalling pathway. Dark-grown *gun1* seedlings are defective in greening after illumination compared with the wild type (Susek *et al*., 1993; Mochizuki *et al*., 1996). A collection of putative *gun1*-like mutants has been isolated by their ability to express photosynthesis genes in the presence of norflurazon (an inhibitor of carotenoid biosynthesis resulting in photooxidation of plastids and down-regulation of nuclear photosynthesis genes in wild-type seedlings) or lincomycin (Gray *et al*., 2003; Cottage *et al*., 2008). Several putative *gun1*-like mutants were examined for their greening phenotype, and one of the lines was able to green on illumination after prolonged darkness. This report describes some of the characteristics of the mutant, suggesting it has an altered sensitivity to ABA.

Materials and methods

Plant materials

Seeds of wild-type *Arabidopsis thaliana* ecotypes, Columbia (Col-0) and Wassilewskaja (Ws), and the *ged1* mutant were obtained from the laboratory stock of the Molecular Biology Group, Department of Plant Sciences, University of Cambridge, UK. *ged1* had initially been isolated as a putative *gun1*-like mutant, PR48.2N, from a mutagenesis experiment (Gray *et al*., 2003). A transgenic *Arabidopsis* Ws line containing reporter genes, green fluorescent protein (GFP) and *Escherichia coli aphIV* (hygromycin resistance) genes, under the control of a tobacco *RbcS* promoter had been mutagenized with ethyl methanesulfonate (EMS), and a collection of putative *gun1*-like mutants had been isolated by their ability to express GFP in the presence of norflurazon and lincomycin (Gray *et al*., 2003; Cottage *et al*., 2008). Seeds of *gun1-1* (Susek *et al*., 1993; Mochizuki *et al*., 1996) in the Col background were obtained from J Chory (Plant Biology Laboratory and Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA, USA).

Plant growth procedures

Seeds were surface-sterilized by immersion in 70% (v/v) ethanol for 2 min, 10% (v/v) sodium hypochlorite (Fisher Scientific, Loughborough, UK), and 0.5% (v/v) Tween-20 (Sigma, Poole, UK) for 15 min, and then rinsed four times in sterile deionized water. The seeds were spread on 0.7% (w/v) micro agar (Duchefa Biochemie, Haarlem, The Netherlands) containing half-strength MS medium (Murashige and Skoog, 1962; Duchefa Biochemie) in a 9 cm Petri dish. Norflurazon (5 μM) (Sandoz Agro, Des Plaines, IL, USA) and 0.5 mM lincomycin hydrochloride (Duchefa Biochemie) were added to the medium whenever specified. When seedlings were to be used for RNA gel-blot analysis, the seeds were sown onto a sterilized 20 μm nylon mesh circle 8.5 cm in diameter (Normesh, Oldham, UK) overlaid onto the medium. The seeds were stratified at 4 °C in the dark (Petri dish wrapped in two layers of aluminium foil) overnight and then grown at 22 °C under a mixture of cool white and GRO-LUX fluorescent lights in a 3:2 ratio (average light intensity 60 μmol m^{-2} s^{-1}, 16 h diurnal photoperiod). For dark treatment, plates were wrapped in two layers of aluminium foil for the indicated periods after being stratified overnight at 4 °C and light treated for 24 h in a 22 °C growth room with a mixture of cool white (Osram, Munich, Germany) and GRO-LUX (Sylvania, Erlangen, Germany) fluorescent lights in a 3:2 ratio (light intensity 60 μmol m^{-2} s^{-1}; 16 h diurnal photoperiod).

For soil-grown seedlings, seeds were sown on a 3:2 (v/v) mixture of Levington M3 (medium structure and high nutrient) compost (Scotts UK Professional, Bramford, UK) and fine vermiculite (William Sinclair Horticulture, Lincoln, UK). The soil mixture was watered with 0.2 g l^{-1} Intercept 70WG (Scotts UK Professional), a systemic and curative insecticide that gives protection from and control of some common pests. The seeds were stratified at 4 °C for 3 d and removed to a 22 °C growth room with alternating cool white and GRO-LUX fluorescent lights (light intensity 40 μmol m^{-2} s^{-1}; 16 h diurnal photoperiod).

Pigment analysis

Chlorophyll was extracted using a protocol modified from that described in Moran and Porath (1980). Approximately 200 mg of

seedlings was immersed in 1.5 ml of *N*,*N*-dimethylformamide (DMF) for 24 h at 4 °C in complete darkness (wrapped with aluminium foil). The extract was subjected to spectrophotometric measurements at 664, 647, 625, and 603 nm using a UV/VIS Spectrometer Lambda 9 (PerkinElmer, Beaconsfield, UK). Total chlorophyll was calculated using equations described by Moran (1982) and standardized to the fresh weight of seedling tissue (μg of chlorophyll g^{-1} of seedling fresh weight).

Transmission electron microscopy

The process was performed as described in Peracchia and Mittler (1972) with modifications. Seedlings were fixed by immersion in 0.1 M PIPES buffer (pH 7.4) containing 4% (v/v) glutaraldehyde, 0.3% (v/v) hydrogen peroxide, and 2 mM $CaCl_2$. Tissues were fixed for 4–6 h at 4 °C, washed twice in buffer (0.1 M PIPES, pH 7.4), and stored at 4 °C. After buffer washes, tissues were post-fixed in 1% (w/v) osmium ferricyanide for 1 h, rinsed three times in deionized water, and stained in 2% (w/v) uranyl acetate for 1 h. Tissues were rinsed in deionized water and dehydrated in an ascending series of ethanol solutions to absolute ethanol [3× 70% (v/v), 3× 95% (v/v), and 3× 100% (v/v)], rinsed twice in acetonitrile, and embedded in Spurr's epoxy resin. Sections (50 nm) were cut on a Leica Ultracut UCT, stained with saturated uranyl acetate in 50% (v/v) ethanol and lead citrate (Reynolds, 1963), and viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV. The entire process of fixation, sectioning, staining, and viewing was performed at the Multi-Imaging Centre, Department of Anatomy, University of Cambridge, UK.

RNA extraction and gel-blot analysis

RNA was prepared from plant materials with Concert Plant RNA Reagent (Invitrogen, Paisley, UK) using the manufacturer's large-scale isolation protocol with additional steps. Before the isopropanol precipitation, the colourless aqueous upper layer was transferred to a 15 ml tube and 1 vol. of water-saturated phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] was added and vortexed. The mixture was centrifuged at 3500 *g* for 15 min at 4 °C. The supernatant was transferred to a fresh tube and the phenol/chloroform/isoamyl alcohol extraction steps were repeated for another two rounds. Total RNA (7–15 μg) was separated by electrophoresis on formaldehyde–1.2% (w/v) agarose gels and then blotted onto a GeneScreen Plus hybridization transfer membrane (PerkinElmer) by capillary blotting (Alwine *et al*., 1977; Helliwell *et al*., 1997).

Radiolabelled probes, prepared by the random-primer method (Feinberg and Vogelstein, 1983) using random hexanucleotide primers and [α-^{32}P]dATP (40 μCi/1.48 MBq; Amersham Biosciences, Little Chalfont, UK), were produced from DNA fragments generated either by restriction enzyme digest from plasmids or by PCR from cDNA. The 509 bp probe for *ACT* [generated from *ACT7* (At5g09810); predicted to cross-hybridize with other *ACT* genes] was excised from a plasmid, pCR-Blunt-ArabACTIN, produced by inserting the probe fragment into a pCR-Blunt vector (Invitrogen), obtained from NJ Brown (Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, UK). Probes for *CRA1* (At5g44120), *HEMA* (At1g58290), *LEA76* (At3g15670), *LHCB1* (At1g29910), *OLEO2* (At5g40420), and *RBCS* (At1g67090) were generated from cDNA derived from mature *Arabidopsis* leaves or dark-grown seedlings by PCR with the primers listed in Supplementary Table S1 available at *JXB* online. Hybridizations with each of the radiolabelled probes were performed at 42 °C and the blots were washed in 2× SSC for 15 min at room temperature, 2× SSC containing 1% (w/v) SDS for 15 min at 65 °C, and 0.1× SSC for 15 min at 65 °C. The membranes were wrapped in Saran film, placed on a Phosphor Screen (Molecular Dynamics; Amersham Biosciences), and exposed for 2 h to 3 d. An image was then produced using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics; Amersham Biosciences). Hybridization signals were quantified using ImageQuant (Molecular Dynamics; Amersham Biosciences). Probes were stripped from membranes by washing with a boiling solution of 0.1× SSC containing 1% (w/v) SDS for 15 min and rinsing with 2× SSC. Membranes were then re-probed.

Affymetrix GeneChip expression analysis

Total RNA for the analysis was extracted, as described above, from seedlings grown for 5 d in the dark followed by 2 d in the light, and cleaned with MicroCon YM-30 columns (Millipore, Watford, UK). The entire process of biotin-labelled cRNA preparation using an Enzo BioArray HighYield RNA Transcript Labelling Kit, hybridization to Affymetrix ATH-121501 microarrays, staining and washing on the fluidics station, array scanning, and data normalization (global scaling) was carried out by the Medical Research Council (MRC) GeneService at Hinxton Hall, Cambridge, UK, according to standard Affymetrix protocols. Data were returned as text files and analysed using GeneSpring 7 (Silicon Genetics, San Carlos, CA, USA). Three replicates for each test line were performed, and average values of transcript amounts were used. Correspondence between the Affymetrix probe set ID, Arabidopsis Genome Initiative (AGI) codes, and the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) mRNA ID was determined using Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/). Microarray data sets were deposited in the Gene Expression Omnibus (GEO) public database with series accession number GSE12401.

In order to search for common *cis*-regulatory sequences in upstream regions of highly affected genes, the 'Find Potential Regulatory Sequences' tool in GeneSpring was used based on the following criteria: 6–8 nucleotides without any point discrepancies within 10–1000 nucleotides upstream of the translation start site of each gene. A cut-off *P*-value of 1×10^{-10} was used in all statistical analysis relative to the upstream region of other (unselected) genes. Sequences of the *A. thaliana* genome were downloaded from the NCBI Genome database (ftp://ftp.ncbi.nih.gov/genomes/Arabidopsis_thaliana/).

Measurement of endogenous ABA

The concentration of ABA in seedling tissue was determined by radioimmunoassay as initially described in Quarrie *et al*. (1988). Seedlings (500–1000) were ground to a fine powder in a mortar with a pestle in liquid nitrogen. The powder was freeze-dried using an Edwards Super Modulyo Freeze-dryer. The powder was extracted with distilled water at a ratio of 20:1 (water volume:seedling dry weight) at 5–10 °C overnight. Each sample (50 μl) was incubated for 45 min with 100 μl of DL-*cis*,*trans*-[G-^{3}H]ABA (Amersham Biosciences) and 100 μl of MAC 252 monoclonal antibody against (S)-*cis*,*trans*-ABA (obtained from GW Butcher, Babraham Institute, Cambridge, UK). Excess label was removed by washing the bound complex twice with 100% and then 50% saturated ammonium sulphate. The pellet was resuspended in 100 μl of water with 1.5 ml of Ecoscint-H scintillation cocktail (National Diagnostics, Hull, UK) for counting (Tri-Carb 1600TR, Packard Instrument Company, Meriden, CT, USA). The concentration of ABA in samples was calculated by interpolation of radioactive counts from a curve of standards that had been linearized by plotting the logit-transformation of the data against the natural logarithm of the amount of unlabelled ABA. All

measurements of ABA concentration were normalized to the dry weight of seedling tissue (μg ABA g^{-1} seedling dry weight). Three replicates were prepared, and 2–4 measurements were performed for each replicate. All the measurements for a replicate were averaged.

Germination experiment

ABA (±*cis-trans*; Sigma A1049) was dissolved in 1 M NaOH and diluted in sterile deionized water to a final stock concentration of 25 mM. For seed germination assay on ABA, 25–150 seeds were sterilized and plated on 0.7% (w/v) agar containing half-strength MS and ABA of different concentrations as indicated in the text. The seeds were stratified at 4 °C in the dark and transferred to the light. Controls without ABA in the medium were prepared in a separate room to avoid possible contamination. After 10 d, the number of seeds showing radicle emergence observable to the naked eye was determined.

Results

Isolation of the greening after extended darkness 1 (ged1) *mutant*

In an attempt to isolate more *gun1*-like mutants that express nuclear photosynthesis genes in the presence of norflurazon or lincomycin (inhibitors of plastid function), a transgenic *Arabidopsis* Ws line showing expression of green fluorescent protein (GFP) under the control of a tobacco *RbcS* promoter had been mutagenized with EMS, and a collection of putative *gun1*-like mutants had been isolated by their ability to express GFP in the presence of norflurazon or lincomycin (Gray *et al*., 2003; Cottage *et al*., 2008). One of the putative *gun1*-like mutant lines, PR48.2N, was able to green on illumination after prolonged darkness.

The greening ability of etiolated PR48.2N seedlings was compared with that of seedlings of the *gun1-1* mutant. The chlorophyll content of seedlings of *gun1-1* and PR48.2N grown for 6 d or 10 d in the dark followed by 1 d in the light was determined after extraction with DMF. For seedlings illuminated after being grown in the dark for 6 d, *gun1-1* accumulated much less total chlorophyll (0.3 μg g^{-1}) than its wild type, Col (13.9 μg g^{-1}) (Fig. 1A). This was consistent with the previous observation that *gun1-1* is defective in greening after the transition from dark to light (Susek *et al*., 1993; Mochizuki *et al*., 1996). In contrast, PR48.2N seedlings accumulated much more total chlorophyll (62.2 μg g^{-1}) than its wild type, Ws (16.1 μg g^{-1}), on illumination. For seedlings illuminated after growth in the dark for 10 d, *gun1-1* contained a similar amount of chlorophyll to its wild type, whereas PR48.2N seedlings accumulated more total chlorophyll (6.4 μg g^{-1}) compared with the wild-type Ws (1.4 μg g^{-1}). PR48.2N seedlings showed an enhanced greening ability after prolonged periods of darkness, and therefore the mutant was named *greening after extended darkness 1* (*ged1*).

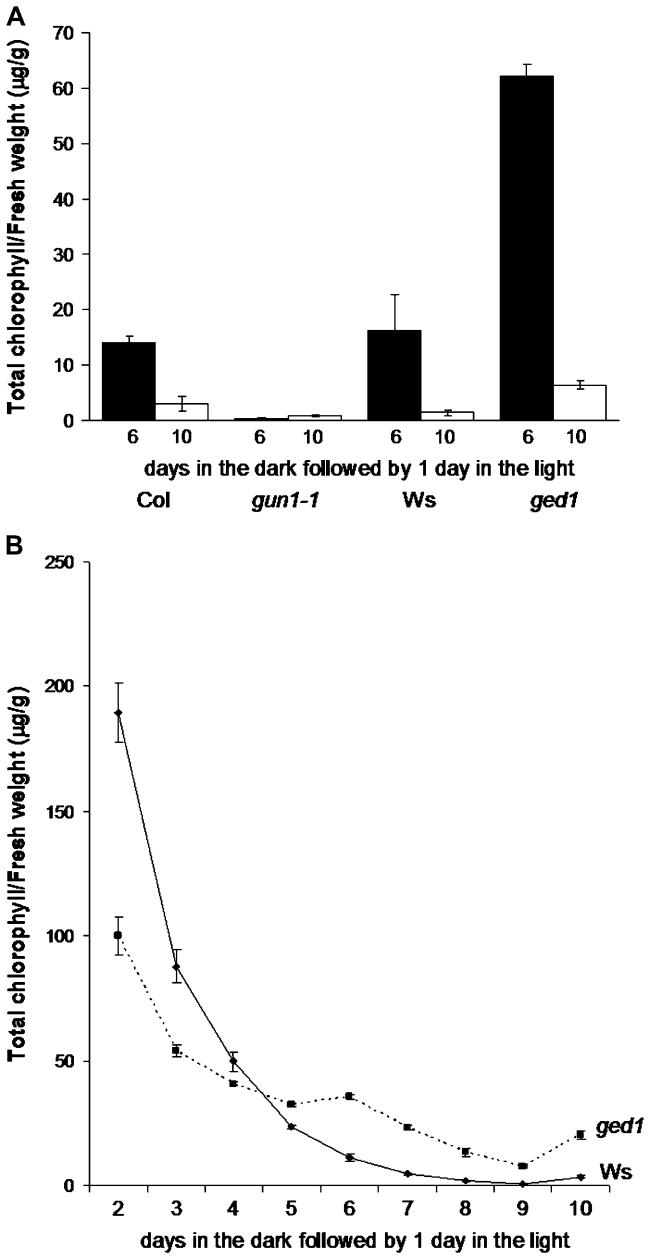

Fig. 1. Chlorophyll content of *Arabidopsis* seedlings grown in various periods of darkness followed by 1 d in the light. (A) Seedlings of *gun1-1* and *ged1* mutants with their respective wild types Col-0 and Ws were grown on 0.7% agar with half-strength MS for 6 d (dark bars) or 10 d (white bars) in the dark followed by 1 d in the light. Chlorophylls were extracted using *N*,*N*-dimethylformamide (DMF) and measured with a UV/VIS Spectrometer Lambda 9. Total chlorophyll was calculated using an equation described by Moran (1982) on the basis of seedling fresh weight. (B) Chlorophyll content of wild-type Ws (solid line) and *ged1* (dotted line) seedlings was measured after 2–10 d in the dark followed by 1 d in the light. Results are means ±SEM for three replicates.

In order to examine the effect of the length of the dark period on chlorophyll accumulation, a time-course experiment was performed with wild-type Ws and *ged1* seedlings grown for 2–10 d in the dark followed by 1 d

in the light. The amount of chlorophyll accumulated in illuminated seedlings of both wild-type Ws and *ged1* decreased with increasing lengths of the dark treatment (Fig. 1B). However, the amount of chlorophyll accumulated by illuminated *ged1* seedlings decreased ~5-fold from 2 d to 10 d in the dark, whereas illuminated wild-type seedlings contained ~40-fold less chlorophyll over the same period. Illuminated *ged1* seedlings accumulated less chlorophyll than the wild type after 2–4 d in the dark, but accumulated more chlorophyll after 5–10 d in the dark. The greening phenotype of *ged1* is clearly different from that of *gun1-1*, which showed reduced greening after extended darkness (Susek *et al*., 1993; Mochizuki *et al*., 1996).

Plastid ultrastructure is altered in ged1 *during the greening process*

Since *ged1* had an altered pattern of chlorophyll accumulation following illumination of dark-grown seedlings, the ultrastructure of plastids of wild-type Ws and *ged1* during the greening process was investigated using transmission electron microscopy. In seedlings grown in continuous darkness for 2 d, prolamellar bodies and primary thylakoids were observed in the etioplasts present in the cotyledons of both wild-type Ws and *ged1* seedlings (Fig. 2A, B). However, etioplasts in *ged1* cotyledons were relatively smaller than the wild-type etioplasts. Following illumination of seedlings grown for 2 d in the dark, chloroplasts were developed with thylakoid membranes and starch grains in wild-type Ws cotyledons (Fig. 2C), whereas in *ged1* cotyledons, chloroplasts with fewer thylakoid membranes and almost devoid of starch grains were observed (Fig. 2D). This observation correlated with the lower chlorophyll accumulation in *ged1* seedlings compared with the wild-type Ws seedlings.

For seedlings grown in continuous darkness for 6 days, wild-type Ws and *ged1* cotyledons contained similar etioplasts with typical prolamellar bodies at the centre (Fig. 2E, F). The smaller etioplasts observed in 2-day-old dark-grown *ged1* seedlings appeared to have increased to wild-type size after 6 d. After illumination, wild-type Ws and *ged1* chloroplasts in the cotyledons showed similar structures. The chloroplasts of wild-type Ws and *ged1* cotyledons contained fewer thylakoid membranes compared with the wild-type chloroplasts after 2 d in the dark followed by 1 d in the light, and were almost devoid of starch grains (Fig. 2G, H). This correlates with the much lower chlorophyll content of both wild-type Ws and *ged1* seedlings after 6 d in the dark followed by 1 d in the light, compared with the wild-type Ws seedlings after 2 d in the dark followed by 1 d in the light.

In seedlings grown in continuous darkness for 10 d, etioplasts of *ged1* cotyledons (Fig. 2J) were similar, although smaller, to the wild-type etioplasts after 2 d in the dark (Fig. 2A), which contained prolamellar bodies and primary thylakoids. Etioplasts of wild-type Ws cotyledons after 10 d in the dark were different from those in *ged1*. The etioplasts showed some rudimentary

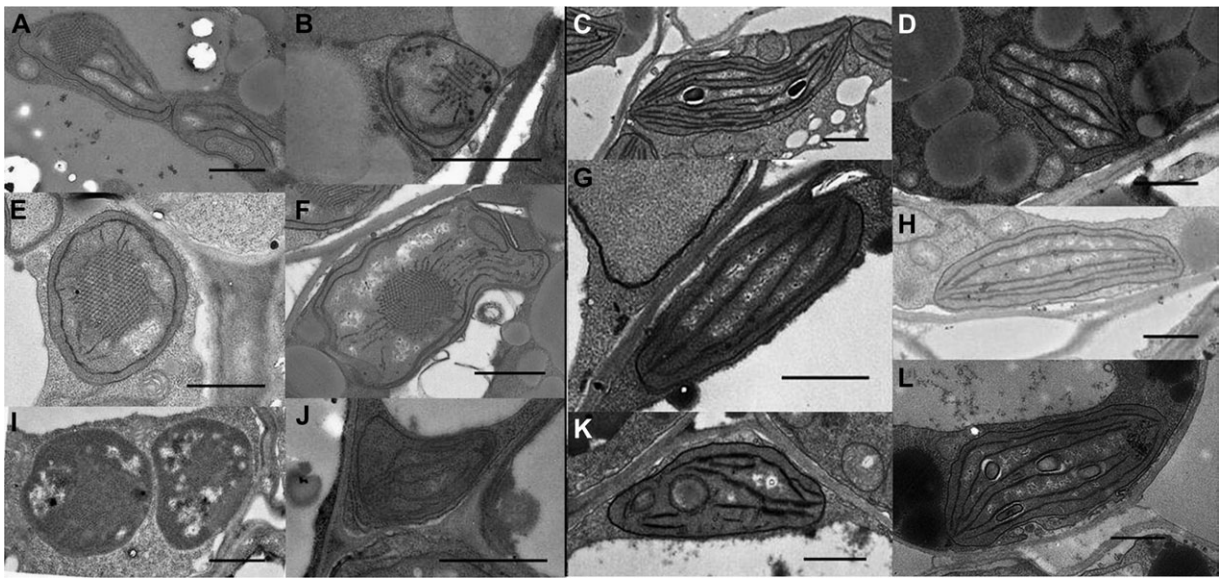

Fig. 2. Transmission electron microscopy of plastids from cotyledons of wild-type Ws and *ged1* seedlings. Wild-type Ws and *ged1* seedlings were grown on 0.7% agar with half-strength MS for 2, 6, or 10 d in the dark and 2, 6, or 10 d in the dark followed by 1 d in the light. Seedlings were fixed in glutaraldehyde and hydrogen peroxide, embedded in Spurr's epoxy resin, and stained with uranyl acetate and lead citrate before being viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV. Bars=1 μm. (A, E, and I) Plastids from cotyledons of wild-type Ws seedlings grown for (A) 2, (E) 6, and (I) 10 d in the dark. (B, F, and J) Plastids from cotyledons of *ged1* seedlings grown for (B) 2, (F) 6, and (J) 10 d in the dark. (C, G, and K) Plastids from cotyledons of wild-type Ws seedlings grown for (C) 2, (G) 6, and (K) 10 d in the dark followed by 1 d in the light. (D, H, and L) Plastids from cotyledons of *ged1* seedlings grown for (D) 2, (H) 6, and (L) 10 d in the dark followed by 1 d in the light.

structures (Fig. 2I) and were similar to wild-type *Arabidopsis* etioplasts observed in plants grown in the dark for 21 d (Rohde *et al*., 2000). In illuminated seedlings after 10 d in the dark, chloroplasts in *ged1* cotyledons contained more thylakoid membranes and starch grains than wild-type Ws seedlings (Fig. 2K, L). The wild-type etioplasts may be in an arrested state after being too long in the dark because they did not differentiate into fully developed chloroplasts (Fig. 2K) and the seedlings did not green properly. However, the number of thylakoid membranes in *ged1* chloroplasts was lower than that found in wild-type chloroplasts after 2 d in the dark followed by 1 d in the light. This correlates with the observation that total chlorophyll of illuminated *ged1* seedlings after 10 d in the dark did not recover to the wild-type level at 2 d in the dark followed by 1 d in the light. Interestingly, *ged1* chloroplasts were almost devoid of starch grains after 2 d and 6 d in the dark followed by 1 d in the light, but starch grains were observed after 10 d in the dark followed by 1 d in the light.

Abundance of transcripts encoding seed proteins is lower in ged1 *seedlings*

Microarray analysis, which is able to make genome-wide comparisons of transcript abundance between samples, was conducted with total RNA extracted from wild-type Ws and *ged1* seedlings grown for 5 d in the dark followed by 2 d in the light using Affymetrix ATH-121501 arrays. The growth condition has been used previously in the laboratory for the study of plastid signalling in tobacco, pea, and *Arabidopsis* (Gray *et al*., 1995, 2003; Sullivan and Gray, 1999, 2002; Brown *et al*., 2005). Although transcripts of most genes were similar between the two samples, many genes showed fewer transcripts in *ged1* seedlings relative to the wild type (Fig. 3A). For these affected genes, 3482 gene probes detected 2-fold lower transcripts in *ged1* seedlings relative to the wild type, whereas 808 gene probes detected more transcripts in *ged1* than the wild type. Lists of the 50 *Arabidopsis* genes with the highest and 50 with the lowest ratios of Affymetrix values representing transcript amounts in *ged1* seedlings relative to the wild-type are provided as Supplementary data at *JXB* online (Tables S2 and S3).

Transcripts of genes involved in late embryogenesis and seed maturation, including genes encoding seed storage proteins, oleosins, and late embryogenesis abundant (LEA) proteins, were much lower in *ged1* seedlings. Eight gene probes representing genes encoding seed storage proteins, including both 12S globulins and 2S albumins, showed very low transcript amounts in *ged1* seedlings relative to wild-type Ws. For gene probes representing eight genes encoding oleosin proteins, all except one showed at least 3-fold lower transcript levels in *ged1*. Most (11 out of 13) of the *LEA* gene probes detected lower amounts of transcripts in *ged1* seedlings (Fig, 3B; Supplementary Table S4 at *JXB* online). However, transcripts of genes encoding photosynthesis-related proteins, represented by 75 gene probes, and genes encoding proteins involved in chlorophyll biosynthesis, represented by 61 gene probes, were generally similar in both *ged1* and wild-type Ws seedlings (Fig. 3C, D; Supplementary Tables S5, S6 at *JXB* online).

Since *ged1* had initially been isolated as a putative *gun1*-like mutant, RNA gel-blot analysis was performed to examine the effect of light and plastid inhibitors on transcripts encoding photosynthesis-related and seed proteins in *ged1* seedlings. *ged1* seedlings showed 2-fold higher transcript abundance of *RBCS* and *LHCB1* genes compared with the wild type after being grown for 5 d in the dark followed by 2 d in the light (Fig. 4). *ged1* seedlings also contained slightly higher amounts of transcripts of nuclear photosynthesis-related genes, compared with the wild type, after treatments with 5 μM norflurazon or 0.5 mM lincomycin (replicates for *RBCS* and *LHCB1* blots not shown). Although this probably accounts for the isolation of *ged1* from the mutant screen, it is not clear if *ged1* is a true *gun* mutant; *ged1* may be an overexpresser of nuclear photosynthesis-related genes under all the treatments. Wild-type Ws seedlings showed lower amounts of transcripts of *RBCS* and *LHCB1* after treatments with norflurazon or lincomycin and in darkness. This is consistent with previous findings that these photosynthesis genes are regulated by light (Karlin-Neumann *et al*., 1988; Dedonder *et al*., 1993; reviewed by Thompson and White, 1991) and plastid signals (reviewed by Gray *et al*., 2003; Nott *et al*., 2006).

ged1 showed lower amounts of *CRA1* transcripts encoding cruciferin A, a 12S seed storage protein (Pang *et al*., 1988), compared with the wild-type Ws in all treatments. Illuminated *ged1* seedlings after 5 d in the dark contained ~4-fold fewer *CRA1* transcripts compared with the illuminated Ws seedlings, whereas dark-grown *ged1* seedlings contained ~6-fold fewer *CRA1* transcripts than dark-grown wild-type seedlings. In wild-type Ws, there were ~3 times more *CRA1* transcripts in dark-grown seedlings than in light-grown seedlings.

Developing seeds of ged1 *contain wild-type amounts of transcripts encoding seed proteins*

The lower amounts of transcripts of seed protein genes in *ged1* seedlings might be a consequence of decreased expression in developing and mature seeds, rather than during seed germination and seedling development. The transcripts of genes encoding seed storage proteins, LEA proteins, and oleosins were investigated in various stages of seed development of wild-type Ws and *ged1*. Total RNA was isolated from siliques of mature wild-type Ws and *ged1* plants corresponding to four developmental stages: early embryogenesis [1–5 days after flowering (DAF)], maturation (8–11 DAF), late embryogenesis (17–21 DAF),

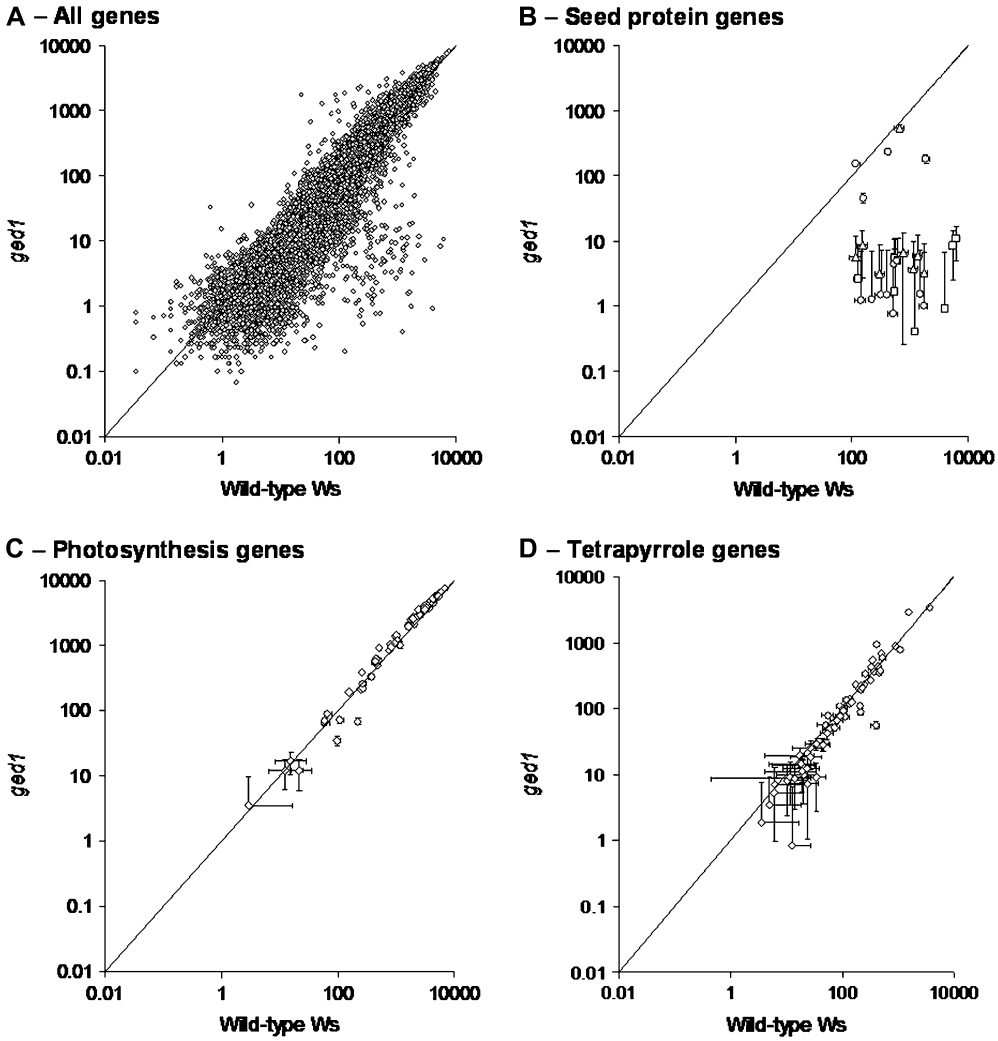

Fig. 3. Genome-wide comparison of transcript levels between wild-type Ws and *ged1* seedlings using Affymetrix microarray analysis. Labelled cRNA, prepared from total RNA extracted from wild-type Ws and *ged1* seedlings grown on 0.7% agar with half-strength MS for 5 d in the dark followed by 2 d in the light, was hybridized to Affymetrix ATH-121501 microarrays. The figure shows the average normalized abundance values of four groups of selected gene transcripts in *ged1* plotted on a log scale against wild-type Ws. (A) Transcript levels of all genes represented by ~23 000 gene probes on the microarrays; (B) transcript levels of genes encoding seed protein genes such as seed storage proteins (open squares, eight gene probes), oleosins (open triangles; eight gene probes), and late embryogenesis abundant proteins (open circles; 13 gene probes); (C) transcript levels of genes encoding photosynthesis proteins represented by 64 gene probes; and (D) transcript levels of genes encoding chlorophyll biosynthesis enzymes represented by 61 gene probes. The diagonal line shows the position expected for gene transcripts that do not change. Results for A are means for three replicates, and results for B, C, and D are means ±SEM for three replicates. Lower limits of error bars with negative values are not shown in the log-scaled scatter plots.

and dry seeds (>21 DAF) (Baud *et al*., 2002), and was subjected to RNA gel-blot analysis using probes for the seed protein genes *CRA1*, *OLEO2* encoding a type 2 oleosin (Zou *et al*., 1996), and *LEA76* encoding an LEA protein 76 homologue (Harada *et al*., 1989). The amounts of transcripts of the seed protein genes were not obviously different between developing or mature seeds of wild-type Ws and *ged1* (Fig. 5).

At 1–5 DAF, corresponding to early embryogenesis, no transcripts were detected for *CRA1*, *OLEO2*, or *LEA76* in either wild-type Ws or *ged1*, in agreement with previous work (Parcy *et al*., 1994). Transcripts of *CRA1* and *OLEO2*, but not *LEA76*, appeared in both wild type and *ged1* at 8–11 DAF, corresponding to seed maturation. *ged1* contained slightly lower amounts of *CRA1* and *OLEO2* transcripts at this stage. At late embryogenesis (17–21 DAF), both the wild type and *ged1* contained transcripts of *CRA1*, *OLEO2*, and *LEA76* genes, and, for dry mature seeds (>21 DAF), transcripts were detected in low quantities for *CRA1*, *OLEO2*, and *LEA76* in both wild-type Ws and *ged1*. The low amounts of *CRA1*, *OLEO2*, and *LEA76* transcripts at >21 DAF could be due to the degradation of total RNA indicated by the rRNAs in Fig. 5. Isolating total RNA of good quality from

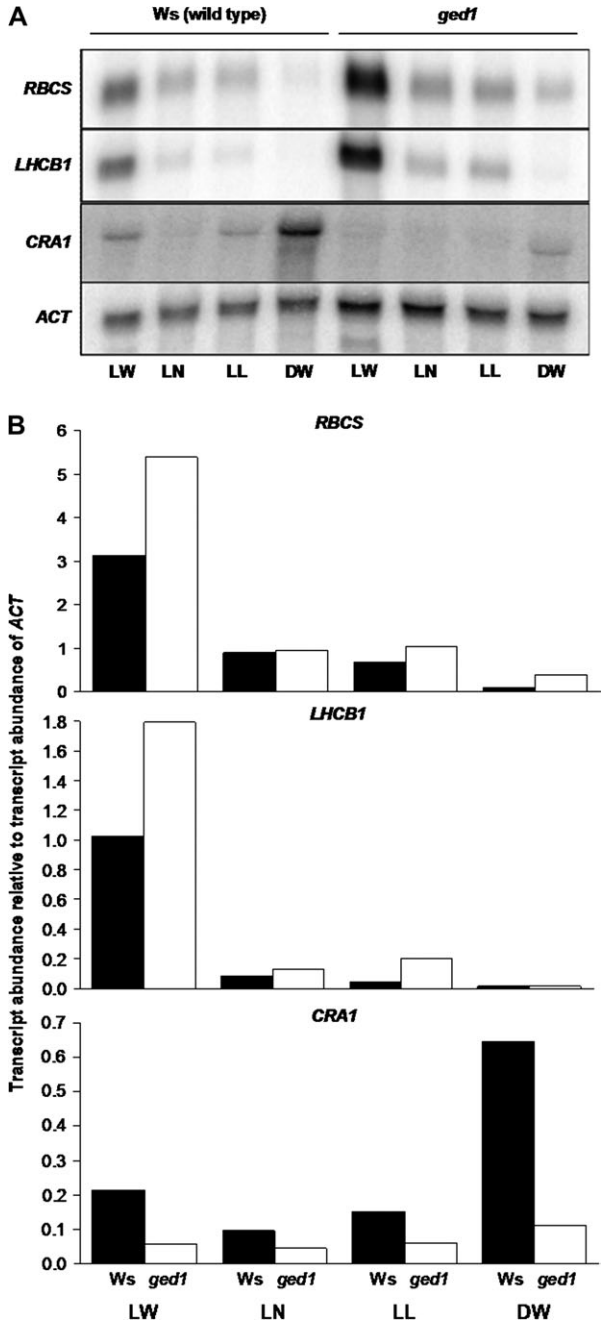

Fig. 4. Effect of light, norflurazon, and lincomycin on the transcript abundance of *CRA1*, a 12S seed storage protein gene, in wild-type Ws, and *ged1* seedlings. (A) Seeds of wild-type Ws and *ged1* were germinated on 0.7% agar with half-strength MS and grown for 5 d in the dark followed by 2 d in the light with either water (LW), 5 μM norflurazon (LN), or 0.5 mM lincomycin (LL) or in continuous darkness for 7 d with water (DW). Total RNA was extracted from the seedlings using Concert Plant RNA Reagent, and 7 μg of the total RNA was subjected to RNA gel-blot analysis using ^{32}P-labelled probes for *RBCS*, *LHCB1*, and *CRA1*, a 12S seed storage protein gene. Signals from a probe for *ACT* hybridized on the blot are shown as loading controls. (B) Hybridization signals from the RNA gel-blots shown in (A) were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardized to *ACT* signals to account for differences in the loading of total RNA. Dark bars=wild-type Ws; white bars=*ged1*.

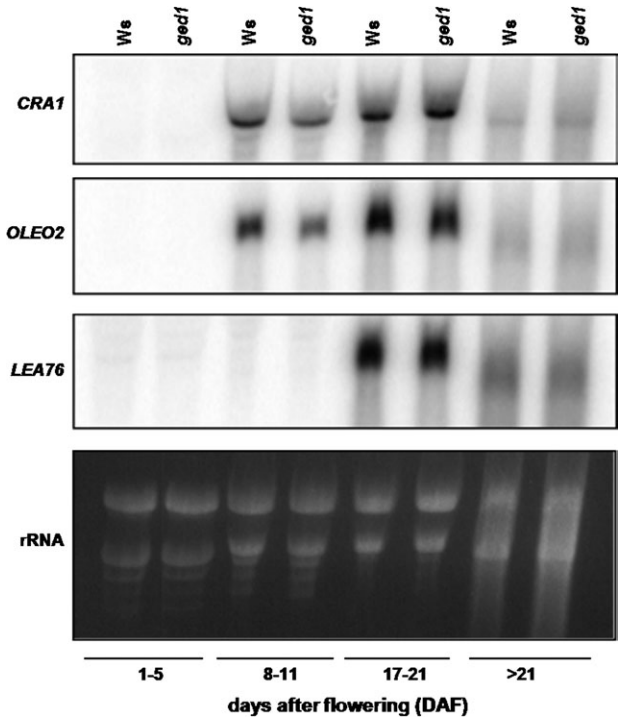

Fig. 5. Transcript abundance of genes encoding a 12S seed storage protein (CRA1), an oleosin type 2 (OLEO2), and a late embryogenesis abundant 76 homologue protein (LEA76) in wild-type Ws and *ged1* at different stages of seed development. Total RNA was extracted from siliques of different stages, namely 1–5, 8–11, and 17–21 days after flowering (DAF) corresponding to early embryogenesis, maturation, and late embryogenesis, and dry seeds (>21 DAF) of wild-type Ws and *ged1* using Concert Plant RNA Reagent, and 15 μg of the total RNA was subjected to RNA gel-blot analysis using ^{32}P-labelled probes from genes encoding CRA1, OLEO2, and LEA76. Ethidium bromide-stained rRNAs are shown as a loading control.

Arabidopsis dry mature seeds is a challenging task (Tai *et al*., 2004). Despite the degradation, the intensity of ethidium bromide-stained rRNA in Fig. 5 indicated that the loading of total RNA was roughly equal. No apparent difference between the wild type and *ged1* was observed in the amounts of transcripts of *CRA1*, *OLEO2*, and *LEA76* during any of the stages of seed development. Hence, the seedling phenotype of *ged1* was unlikely to be a consequence of decreased expression during embryogenesis and seed maturation.

Temporal changes in transcripts of seed protein genes are abolished in illuminated dark-grown ged1 *seedlings*

To examine the effect of different periods of darkness and subsequent illumination on transcripts of nuclear genes encoding photosynthesis and seed proteins, total RNA extracted from Ws and *ged1* seedlings was subjected to RNA gel-blot analysis. Probes for *RBCS*, *LHCB1*, *HEMA* encoding glutamyl-tRNA reductase, a key component of the tetrapyrrole biosynthesis pathway (Ilag *et al*., 1994;

Kumar *et al*., 1996), *CRA1*, *OLEO2*, and *LEA76* were used. The transcripts of nuclear photosynthesis-related and seed protein genes were markedly lower in dark-grown *ged1* seedlings than in dark-grown wild-type seedlings (Figs 6, 7). *HEMA* showed the smallest differences in amounts of transcripts, but showed a transient increase in transcripts after 4 d in the dark in wild-type seedlings but not in *ged1* seedlings. This transient increase after 4 d in the dark was observed in wild-type Ws seedlings for all transcripts examined, but it did not occur in *ged1* seedlings. This transient increase appears to be similar to the transient increase in the amounts of *LHCB1* and *RBCS* transcripts in early development of dark-grown *Arabidopsis* seedlings reported by Brusslan and Tobin (1992).

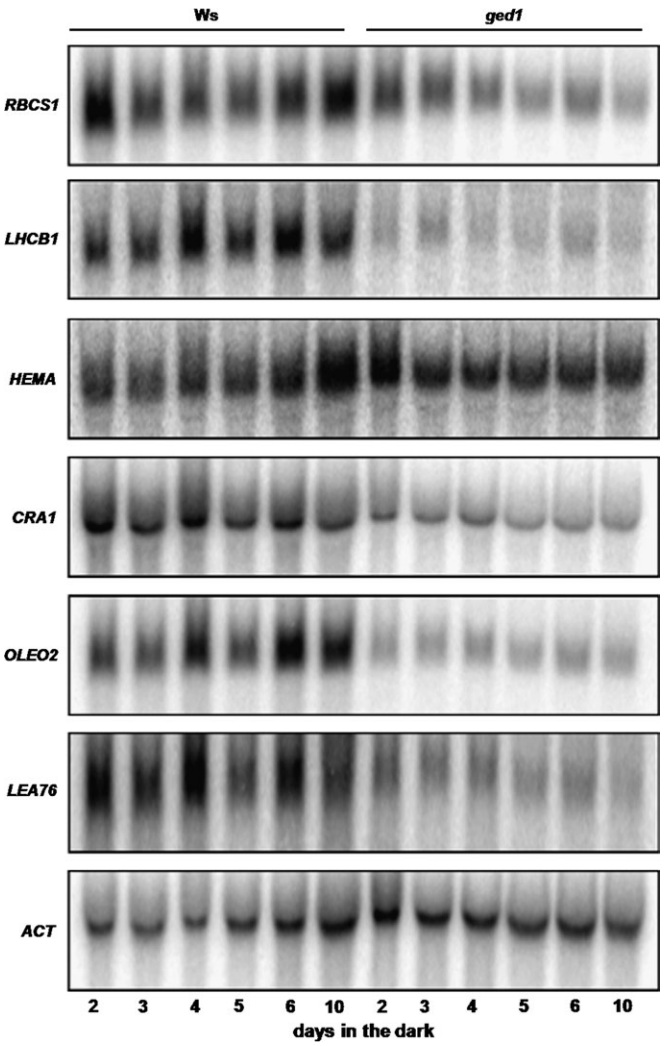

Fig. 6. Transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type Ws and *ged1* seedlings grown in various periods of darkness. Wild-type Ws and *ged1* seedlings were grown on 0.7% agar with half-strength MS for 2, 3, 4, 5, 6, or 10 d in the dark. Total RNA was extracted from the seedlings using Concert Plant RNA Reagent, and 15 μg of the total RNA was subjected to RNA gel-blot analysis using ^{32}P-labelled probes from nuclear genes encoding photosynthesis proteins, RBCS, LHCB1, and HEMA, and seed proteins, CRA1, OLEO2, and LEA76. Signals from a probe for *ACT* hybridized on the blot are shown as loading controls.

There were major differences in the transcripts of seed protein genes, *CRA1*, *OLEO2*, and *LEA76*, between illuminated wild-type and *ged1* seedlings (Figs 8, 9). Very low amounts of transcripts of the seed protein genes were observed in *ged1* seedlings, and there was little change over 2–10 d in darkness. In contrast, there were large changes in transcripts in illuminated wild-type seedlings. Transcripts decreased markedly in illuminated wild-type seedlings after 3–4 d in the dark, with the lowest amounts in illuminated 6-day-old seedlings, where the amounts of transcripts were comparable with those in the *ged1* seedlings. A large increase in the amounts of transcripts of seed protein genes was observed in illuminated 10-day-old wild-type seedlings, but no similar increase was observed in illuminated 10-day-old *ged1* seedlings. The lack of response of transcripts of seed protein genes to changes in seedling development in *ged1* suggests that GED1 may be involved in positive regulation of seed protein gene expression.

The amounts of transcripts of *RBCS*, *LHCB1*, and *HEMA* were comparable between illuminated 2- to 6-day-old wild-type and *ged1* seedlings, but major changes occurred in seedlings on illumination after prolonged darkness (Figs 8, 9). Illuminated 10-day-old wild-type seedlings contained much lower amounts of transcripts of *RBCS*, *LHCB1*, and *HEMA* than younger seedlings, whereas there was not such a marked decrease in these transcripts in illuminated 10-day-old *ged1* seedlings. This resulted in these *ged1* seedlings containing more transcripts of photosynthesis-related genes than wild-type seedlings. This observation may be correlated with the enhanced greening ability of *ged1* seedlings after extended periods of darkness.

ABA response elements (ABREs) are present in upstream regions of genes down-regulated in ged1

From the microarray analysis, 279 NCBI Reference Sequences (RefSeq) that showed at least 10-fold fewer transcripts in *ged1* seedlings compared with the wild type, corresponding to 237 genes listed in Supplementary Table S7 at *JXB* online, were identified, and 1 kb regions upstream of the translation start sites of the genes were searched for common motifs. Seven sequences of possible common *cis*-regulatory elements were found in the upstream regions of the genes showing lower transcripts in *ged1* seedlings relative to wild-type Ws (Table 1). All except one of these sequences contained an ACGT core, and the most prominent sequences were the CACGTG-related elements. CACGTG was observed in 37.6% of the genes analysed. Two extended CACGTG elements, namely ACACGTG and CACGTGT, showed frequencies of 23.7% and 22.9%, respectively. The ACGT-containing elements are similar to ABREs (Guiltinan *et al*., 1990;

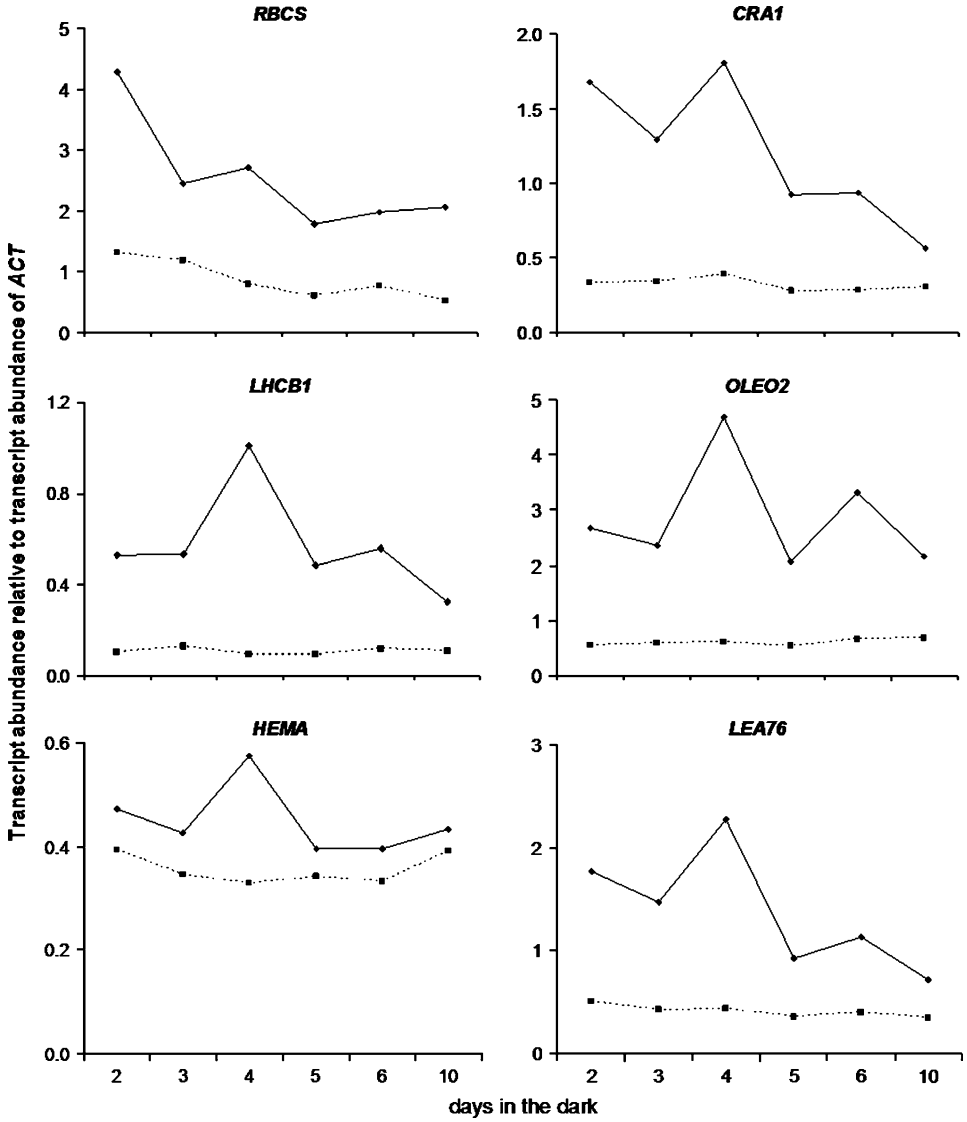

Fig. 7. Quantification of the transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type Ws and *ged1* seedlings grown in various periods of darkness. Hybridization signals from the RNA gel blots in Fig. 6 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardized to *ACT* signals to account for differences in the loading of total RNA. Solid lines=wild-type Ws; dotted lines=*ged1*.

Skriver *et al*., 1991; Shen *et al*., 1993), which contain an ACGT core (Michel *et al*., 1993). The prominent CACGTG-related elements are similar to one of the most typical ABREs previously identified by promoter and binding assays (Guiltinan *et al*., 1990; Michel *et al*., 1993; Shen *et al*., 1993). One element, CGTGTC, identified from the promoter analysis did not contain an ACGT core. CGTGTC was found in 27.6% of the genes analysed. This element shares a common sequence with coupling element 3 (CE3; ACGCGTGTCCTC), which functions in concert with the ACGT-containing ABRE to regulate ABA-inducible gene expression (Kao *et al*., 1996; Shen *et al*., 1996; Hobo *et al*., 1999).

About 60% of the 279 genes examined contained at least one of the elements listed in Table 1. Almost half (47.0%) of the 279 genes contained CACGTG or/and CGTGTC elements in the upstream regions. Half the genes (47.3%) with CACGTG or/and CGTGTC elements in the upstream regions contained only one copy of either one of the elements, whereas the other half (52.7%) contained multiple copies of either one of the elements or combinations of both elements in various numbers (data not shown). Using the same search criteria, no common *cis*-regulatory elements were found in the upstream regions of genes that did not contain CACGTG or CGTGTC elements.

Germination of ged1 *is hypersensitive to ABA*

The identification of ABREs in the upstream region of many of the genes affected in *ged1* suggested that ABA

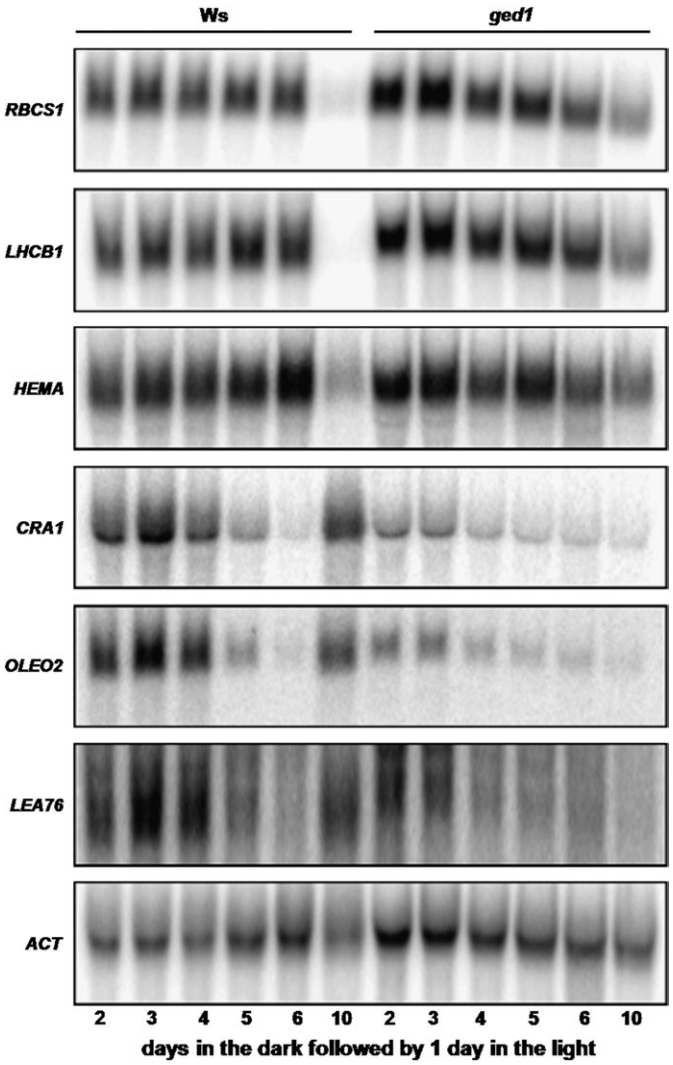

Fig. 8. Transcript abundance of nuclear genes encoding photosynthesis and seed proteins in wild-type Ws and *ged1* seedlings grown in various periods of darkness followed by 1 d in the light. Wild-type Ws and *ged1* seedlings were grown on 0.7% agar with half-strength MS for 2, 3, 4, 5, 6, or 10 d in the dark followed by 1 d in the light. Total RNA was extracted from the seedlings using Concert Plant RNA Reagent, and 15 μg of the total RNA was subjected to RNA gel-blot analysis using ^{32}P-labelled probes from nuclear genes encoding photosynthesis proteins, RBCS, LHCB1, and HEMA, and seed proteins, CRA1, OLEO2, and LEA76. Signals from a probe for *ACT* hybridized on the blot are shown as loading controls.

regulation of gene expression may be perturbed in *ged1*. Expression of seed storage protein or LEA protein genes is down-regulated in developing seeds of *Arabidopsis* mutants deficient in ABA biosynthesis or insensitive to ABA (Koornneef *et al*., 1989; Kriz *et al*., 1990; Meurs *et al*., 1992; Finkelstein, 1993; Paiva and Kriz, 1994; Parcy *et al*., 1994). In order to establish if *ged1* was a mutant deficient in ABA biosynthesis, the ABA content of wild-type Ws and *ged1* seedlings grown for 5 d in the dark followed by 2 d in the light was measured by radioimmunoassay. The ABA content of wild-type Ws samples was 0.36–0.40 μg ABA g^{-1} seedling dry weight, whereas for *ged1* samples, the range was 0.38–0.43 μg g^{-1}. These values were not significantly different, indicating that *ged1* was not a mutant in ABA biosynthesis, at least in 7-day-old seedlings. The mutant phenotypes of the developing *ged1* seedlings were therefore unlikely to be related to their endogenous ABA level.

To examine if *ged1* seedlings had an altered sensitivity to ABA, wild-type Ws and *ged1* seeds were allowed to germinate on agar medium containing 0–10 μM ABA for 10 d in the light and radicle emergence observable to the naked eye was scored (Fig. 10). Germination of wild-type Ws seeds was markedly inhibited at ABA concentrations of $\geqslant$5 μM, whereas germination of *ged1* seeds was inhibited by 1 μM ABA. The greatest difference in germination between wild-type Ws and *ged1* seeds was observed in the presence of 2.5 μM ABA. At this concentration, full germination of wild-type seeds was obtained, whereas only ~10% of *ged1* seeds germinated. This 10-fold difference in germination in the presence of 2.5 μM ABA clearly indicated that *ged1* was hypersensitive to the inhibitory effect of ABA on germination.

Discussion

We have isolated an *Arabidopsis* mutant, *ged1* (*greening after extended darkness1*), showing a suite of phenotypes that can all be related to an altered responsiveness to ABA. The mutant was originally isolated in a screen for *genomes uncoupled* (*gun*) mutants showing increased expression from a tobacco *RbcS* promoter in seedlings treated with norflurazon, which normally represses expression of photosynthesis-related nuclear genes. However, the mutant also showed higher expression of *RBCS* and *LHCB1*, compared with the wild type, in the absence of norflurazon, suggesting that it was an overexpressing mutant and not a true *gun* mutant. Unlike *gun1*, which is defective in greening after transfer of seedlings from dark to light (Mochizuki *et al*., 1996), the mutant seedlings showed an enhanced ability to green on illumination after extended growth in darkness. The mutant did not show the degeneration of etioplast morphology that has been linked to dark-induced increases in ABA shown in wild-type seedlings (Weatherwax *et al*., 1996; Rohde *et al*., 2000). In addition, the extent and patterns of accumulation of transcripts of ABA-regulated genes were markedly different in the mutant compared with wild-type seedlings during these greening experiments. The mutant also showed hypersensitivity to the inhibitory effects of ABA on seed germination. However, mutant seedlings contained wild-type amounts of ABA, suggesting that the mutant phenotypes may be due to altered responsiveness to ABA.

Perhaps the most remarkable of the phenotypes of *ged1* is the much lower amounts of transcripts of seed protein

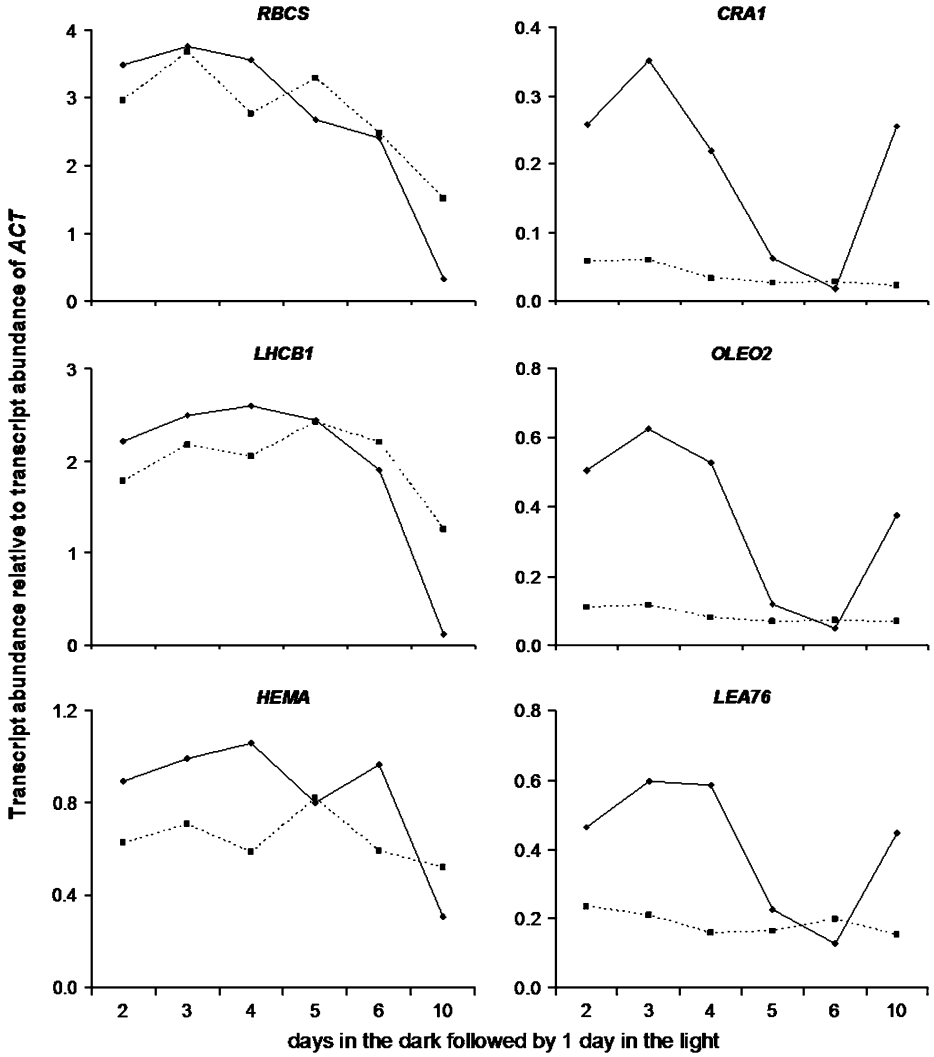

Fig. 9. Quantification of the transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type Ws and *ged1* seedlings grown in various periods of darkness followed by 1 d in the light. Hybridization signals from the RNA gel-blots shown in Fig. 8 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardized to *ACT* signals to account for differences in the loading of total RNA. Solid lines=wild-type Ws; dotted lines=*ged1*.

genes in mutant seedlings. Transcripts of genes encoding seed storage proteins, oleosins, and LEA proteins were up to 1000-fold lower in the mutant seedlings compared with wild-type seedlings by microarray analysis. RNA gel-blot analysis did not detect such extreme differences, but only very low amounts of transcripts of *CRA1*, *OLEO2*, and *LEA76* were detected in 2- to 10-day-old *ged1* seedlings. The temporal patterns of transcript accumulation and decline in wild-type seedlings were not observed in *ged1* seedlings. The marked increase in seed protein gene transcripts observed in illuminated 10-day-old wild-type seedlings did not occur in *ged1* seedlings. Conversely, illuminated 10-day-old wild-type seedlings showed a marked decrease in transcripts of photosynthesis-related genes, and this decline was abrogated in *ged1* seedlings. The opposite behaviour of the seed protein genes and the photosynthesis-related genes in these wild-type seedlings could be speculated to mean that the seedlings were returning to a seed-like stage after extended darkness and may be related to ABA levels, which are known to affect inversely the expression of these genes (Medford and Sussex, 1989; Chang and Walling, 1991; Chandler and Robertson, 1994). ABI3, a transcriptional regulator of seed storage protein and LEA gene expression in developing seeds (Fujiwara *et al*., 2002), also affects plastid differentiation in dark-grown *Arabidopsis* seedlings (Rohde *et al*., 2000).

Examination of the promoter sequences of genes highly affected in *ged1* showed that they shared ACGT-containing elements, similar to ABREs (Guiltinan *et al*., 1990; Skriver *et al*., 1991; Michel *et al*., 1993; Shen *et al*., 1993). Elements similar or identical to the ABREs,

Table 1. *Sequences enriched in upstream regions of genes with 10-fold lower transcript levels in* ged1 *seedlings relative to wild-type Ws*

Upstream sequences of genes that showed 10-fold lower transcript abundance in *ged1* seedlings relative to wild-type Ws were subjected to the 'Find Potential Regulatory Sequences' tool in GeneSpring. The criteria for the search were: 6–8 nucleotides without any point discrepancies within 10–1000 nucleotides upstream from the translation start site of each gene. Random rate is the expected percentage of genes having the specific nucleotide combination in the upstream regions if the nucleotide sequence is random. *P*-value is the probability that the occurrences of the specific nucleotide combination in the selected genes came about by chance. The cut-off *P*-value was set at 1×10^{-10}.

Sequence	Observed in the selected genes (%)	Observed in unselected genes (%)	Random rate (%)	*P*-value
ACGTGT	109/279 (39.1)	16.6	15.0	3.8×10^{-15}
CACGTG	105/279 (37.6)	15.7	8.0	7.6×10^{-15}
ACACGTG	66/279 (23.7)	6.8	2.8	1.5×10^{-14}
CACGTGT	64/279 (22.9)	6.8	2.7	4.7×10^{-13}
ACGTGTC	55/279 (19.7)	5.3	2.7	1.6×10^{-12}
CGTGTC	77/279 (27.6)	10.1	7.9	2.1×10^{-12}
ACACGT	102/279 (36.6)	16.9	16.0	3.2×10^{-11}

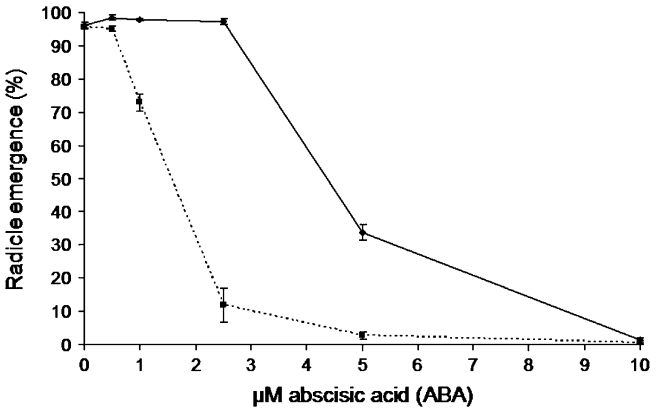

Fig. 10. Dose–response of germination to abscisic acid (ABA) of wild-type Ws and *ged1* seeds after 10 d in the light. Wild-type Ws and *ged1* seeds were sown on 0.7% agar with half-strength MS and various concentrations of ABA, namely 0, 0.5, 1, 2.5, 5, and 10 μM, and allowed to germinate in the light for 10 d. The percentage of seeds showing radicle emergence was determined for >70 seeds. Results are means ±SEM for three replicates. Solid lines=wild-type Ws; dotted lines=*ged1*.

especially the palindromic CACGTG G-box, have also been identified as *cis*-regulatory elements in the promoters of various genes regulated by a variety of environmental and physiological signals (Guiltinan *et al*., 1990; Oeda *et al*., 1991; Williams *et al*., 1992; Michel *et al*., 1993). The presence of a second *cis*-element in the vicinity of the G-box, such as coupling elements CE1 and CE3, has been demonstrated to play an important role to determine the ABA response specificity (Shen and Ho, 1995; Shen *et al*., 1996). However, ABREs and the coupling elements are functionally equivalent as they are interchangeable (Hobo *et al*., 1999) and this is consistent with the fact that only multiple copies of ABRE can confer ABA responsiveness (Shen *et al*., 1993; Shen and Ho, 1995; Vasil *et al*., 1995). Since both ACGT-containing and CGTGTC CE3-like elements were identified in the upstream regions of genes with lower transcript levels in *ged1* seedlings and half of the genes contained multiple copies of either one of the elements or combinations of both, GED1 appears likely to play a role in an ABA-related regulatory pathway.

However, the range of phenotypes exhibited by the *ged1* mutant indicates that *GED1* must play different roles in different tissues at different times. There was no apparent effect of *ged1* on expression of seed protein genes during embryogenesis and seed development, in contrast to the major effects on transcripts of seed protein genes during *ged1* seedling development. Similarly, the *ged1* mutation produced a hypersensitivity of seed germination to exogenous ABA, whereas seed protein gene expression, which is ABA inducible (Chandler and Robertson, 1994), was dramatically decreased in developing *ged1* seedlings. There are a large number of ABA-hypersensitive mutants showing defects in a wide range of plant functions (reviewed in Finkelstein *et al*., 2002). However, no ABA-hypersensitive mutant is known to have similar phenotypes to *ged1*, although *de-etiolated 2* (*det2*), an *Arabidopsis* mutant hypersensitive to ABA, exhibits a light-grown phenotype in the dark, which could be related to the phenotype of enhanced greening ability of *ged1* (Steber and McCourt, 2001). ABI4, a transcriptional regulator, has recently been demonstrated to regulate lipid mobilization in *Arabidopsis* embryos (Penfield *et al*., 2006a) and the expression of photosynthesis-related genes affected by plastid signals (Koussevitzky *et al*., 2007).

Preliminary genetic analysis has confirmed that three of the *ged1* phenotypes (enhanced greening after 10 d of darkness, hypersensitivity of germination to ABA, and lower *CRA1* transcript amounts in seedlings) co-segregate in an F_2 mapping population, suggesting that these phenotypes are the result of a single mutation. The use of CAPS (cleaved amplified polymorphic sequences) and SNP (single nucleotide polymorphism) markers on a small population of mapping individuals suggests that the mutation is likely to be on chromosome 5 (data not shown). Further analysis will be required to identify the mutated gene in *ged1* and to determine its mode of action.

Supplementary data

The following data are available at *JXB* online.

Supplementary Table S1. Probes generated by PCR for RNA gel-blot analysis.

Supplementary Table S2. List of 50 *Arabidopsis* genes with the lowest ratios of transcript amounts (Affymetrix

values) in *ged1* 7-day-old seedlings relative to wild-type Ws seedlings.

SupplementaryTable S3. List of 50 *Arabidopsis* genes with the highest ratios of transcript amounts (Affymetrix values) in *ged1* 7-day-old seedlings relative to wild-type Ws seedlings.

Supplementary Table S4. Affymetrix values representing transcript amounts of seed protein genes in *ged1* and the wild type.

Supplementary Table S5. Affymetrix values representing transcript amounts of photosynthesis genes in *ged1* and wild-type Ws 7-day-old seedlings.

Supplementary Table S6. Affymetrix values representing transcript amounts of chlorophyll biosynthesis in *ged1* and wild-type Ws 7-day-old seedlings.

Supplementary Table S7. List of *Arabidopsis* genes with 10-fold lower transcript levels in *ged1* seedlings relative to wild-type Ws used to search for common *cis*-regulatory sequences in upstream regions.

Acknowledgements

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References

Acevedo-Hernández GJ, León P, Herrera-Estrella LR. 2005. Sugar and ABA responsiveness of a minimal *RBCS* light-responsive unit is mediated by direct binding of ABI4. *The Plant Journal* **43,** 506–519.

Alwine JC, Kemp DJ, Stark GR. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proceedings of the National Academy of Sciences, USA* **74,** 5350–5354.

Baud S, Boutin JP, Miquel M, Lepiniec L, Rochat C. 2002. An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. *Plant Physiology and Biochemistry* **40,** 151–160.

Briggs WR, Christie JM. 2002. Phototropins 1 and 2: versatile plant blue-light receptors. *Trends in Plant Science* **7,** 204–210.

Brosché M, Strid Å. 2003. Molecular events following perception of ultraviolet-B radiation by plants. *Physiologia Plantarum* **117,** 1–10.

Brown NJ, Sullivan JA, Gray JC. 2005. Light and plastid signals regulate the expression of the pea plastocyanin gene through a common region at the 5′ end of the coding region. *The Plant Journal* **43,** 541–552.

Brusslan JA, Tobin EM. 1992. Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings. *Proceedings of the National Academy of Sciences, USA* **89,** 7791–7795.

Cashmore AR, Jarillo JA, Wu YJ, Liu D. 1999. Cryptochromes: blue light receptors for plants and animals. *Science* **284,** 760–765.

Chandler PM, Robertson M. 1994. Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **45,** 113–141.

Chang YC, Walling LL. 1991. Abscisic acid negatively regulates expression of chlorophyll a/b binding protein genes during soybean embryogeny. *Plant Physiology* **97,** 1260–1264.

Chory J, Chatterjee M, Cook RK, *et al.* 1996. From seed germination to flowering, light controls plant development via the pigment phytochrome. *Proceedings of the National Academy of Sciences, USA* **93,** 12066–12071.

Chory J, Reinecke D, Sim S, Washburn T, Brenner M. 1994. A role for cytokinins in de-etiolation in *Arabidopsis*. *Plant Physiology* **104,** 339–347.

Cottage AJ, Mott EK, Wang JH, *et al.* 2008. *GUN1 (GENOMES UNCOUPLED1)* encodes a pentatricopeptide repeat (PPR) protein involved in plastid protein synthesis-responsive retrograde signalling to the nucleus. In: Allen JF, Gantt E, Golbeck JH, Osmond B, eds. *Photosynthesis. Energy from the sun: 14th International Congress on Photosynthesis*. Berlin/Heidelberg: Springer, 1205–1211.

Dedonder A, Rethy R, Fredericq H, Van Montagu M, Krebbers E. 1993. *Arabidopsis rbcS* genes are differentially regulated by light. *Plant Physiology* **101,** 801–808.

Feinberg AP, Vogelstein B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132,** 6–13.

Finkelstein RR. 1993. Abscisic acid-insensitive mutations provide evidence for stage-specific signal pathways regulating expression of an *Arabidopsis* late embryogenesis-abundant (*lea*) gene. *Molecular and General Genetics* **238,** 401–408.

Finkelstein RR, Gampala SSL, Rock CD. 2002. Abscisic acid signaling in seeds and seedlings. *The Plant Cell* **14,** S15–S45.

Fujiwara T, Nambara E, Yamagishi K, Goto DB, Naito S. 2002. Storage proteins. In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists, doi/10.1199/tab.0020, http://www.aspb.org/publications/arabidopsis/.

Gray JC, Sornarajah R, Zabron AA, Duckett CM, Khan MS. 1995. Chloroplast control of nuclear gene expression. In: Mathis P, ed. *Photosynthesis: from light to biosphere,* Vol. 3. Dordrecht: Kluwer Academic Publishers, 543–550.

Gray JC, Sullivan JA, Wang JH, Jerome AA, MacLean D. 2003. Coordination of plastid and nuclear gene expression. *Philosophical Transactions of the Royal Society B: Biological Sciences* **358,** 135–145.

Guiltinan MJ, Marcotte WR, Quatrano RS. 1990. A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* **250,** 267–271.

Harada JJ, DeLisle AJ, Baden CS, Crouch ML. 1989. Unusual sequence of an abscisic acid-inducible mRNA which accumulates late in *Brassica napus* seed development. *Plant Molecular Biology* **12,** 395–401.

Helliwell CA, Webster CI, Gray JC. 1997. Light-regulated expression of the pea plastocyanin gene is mediated by elements within the transcribed region of the gene. *The Plant Journal* **12,** 499–506.

Hobo T, Asada M, Kowyama Y, Hattori T. 1999. ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. *The Plant Journal* **19,** 679–689.

Ilag LL, Kumar AM, Söll D. 1994. Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in Arabidopsis. *The Plant Cell* **6,** 265–275.

Karlin-Neumann GA, Sun L, Tobin EM. 1988. Expression of light-harvesting chlorophyll *a*/*b*-protein genes is phytochrome-regulated in etiolated *Arabidopsis thaliana* seedlings. *Plant Physiology* **88,** 1323–1331.

Kao CY, Cocciolone SM, Vasil IK, McCarty DR. 1996. Localization and interaction of the *cis*-acting elements for abscisic acid, VIVIPAROUS1, and light activation of the *C1* gene of maize. *The Plant Cell* **8,** 1171–1179.

Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM. 1989. *In vivo* inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana*. *Plant Physiology* **90,** 463–469.

Koussevitsky S, Nott A, Mockler TC, Hong F, Sachetto-Martins G, Surpin M, Lim IJ, Mittler R, Chory J. 2007. Signals from chloroplasts converge to regulate nuclear gene expression. *Science* **316,** 715–719.

Kriz AR, Wallace MS, Paiva R. 1990. Globulin gene expression in embryos of maize *viviparous* mutants. Evidence for regulation of the *Glb1* gene by ABA. *Plant Physiology* **92,** 538–542.

Kumar AM, Csankovszki G, Söll D. 1996. A second and differentially expressed glutamyl-tRNA reductase gene from *Arabidopsis thaliana*. *Plant Molecular Biology* **30,** 419–426.

McNellis TW, Deng XW. 1995. Light control of seedling morphogenetic pattern. *The Plant Cell* **7,** 1749–1761.

Medford JI, Sussex IM. 1989. Regulation of chlorophyll and Rubisco levels in embryonic cotyledons of *Phaseolus vulgaris*. *Planta* **179,** 309–315.

Meurs C, Basra AS, Karssen CM, van Loon LC. 1992. Role of abscisic acid in the induction of desiccation tolerance in developing seeds of *Arabidopsis thaliana*. *Plant Physiology* **98,** 1484–1493.

Michel D, Salamini F, Bartels D, Dale P, Baga M, Szalay A. 1993. Analysis of a desiccation and ABA-responsive promoter isolated from the resurrection plant Craterostigma plantagineum. *The Plant Journal* **4,** 29–40.

Mochizuki N, Susek R, Chory J. 1996. An intracellular signal transduction pathway between the chloroplast and nucleus is involved in de-etiolation. *Plant Physiology* **112,** 1465–1469.

Moran R. 1982. Formulae for determination of chlorophyllous pigments extracted with *N*, *N*-dimethylformamide. *Plant Physiology* **69,** 1376–1381.

Moran R, Porath D. 1980. Chlorophyll determination in intact tissues using *N*,*N*-dimethylformamide. *Plant Physiology* **65,** 478–479.

Nemhauser J, Chory J. 2002. Photomorphogenesis. In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists, doi/10.1199/tab.0054, http://www.aspb.org/publications/arabidopsis/.

Nott A, Jung HS, Koussevitzky S, Chory J. 2006. Plastid-to-nucleus retrograde signalling. *Annual Review of Plant Biology* **57,** 739–759.

Oeda K, Salinas J, Chua NH. 1991. A tobacco bZip transcription activator (TAF-1) binds to a G-box-like motif conserved in plant genes. *EMBO Journal* **10,** 1793–1802.

Oelmüller R, Levitan I, Bergfeld R, Rajasekhar VK, Mohr H. 1986. Expression of nuclear genes as affected by treatments acting on plastids. *Planta* **168,** 482–492.

Paiva R, Kriz AL. 1994. Effect of abscisic acid on embryo-specific gene expression during normal and precocious germination in normal and *viviparous* maize (*Zea mays*) embryos. *Planta* **192,** 332–339.

Pang PP, Pruitt RE, Meyerowitz EM. 1988. Molecular cloning, genomic organization, expression and evolution of 12S seed storage protein genes of *Arabidopsis thaliana*. *Plant Molecular Biology* **11,** 805–820.

Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M, Giraudat J. 1994. Regulation of gene expression programs during Arabidopsis seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *The Plant Cell* **6,** 1567–1582.

Penfield S, Li Y, Gilday AD, Graham S, Graham IA. 2006*a*. *Arabidopsis* ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *The Plant Cell* **18,** 1887–1899.

Penfield S, Pinfield-Wells HM, Graham IA. 2006*b*. Storage reserve mobilisation and seedling establishment in Arabidopsis. In: Somerville CR, Meyerowitz EM, eds. *The Arabidopsis Book*. Rockville, MD: American Society of Plant Biologists, doi/10.1199/tab.0100, http://www.aspb.org/publications/arabidopsis/.

Peracchia C, Mittler BS. 1972. Fixation by means of glutaraldehyde–hydrogen peroxide reaction products. *Journal of Cell Biology* **53,** 234–238.

Quail PH, Boylan M, Parks BM, Short TW, Xu Y, Wagner D. 1995. Phytochromes: photosensory perception and signal transduction. *Science* **268,** 675–680.

Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE, Loveys BR. 1988. A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* **173,** 330–339.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17,** 208–212.

Rohde A, De Rycke R, Beeckman T, Engler G, Van Montagu M, Boerjan W. 2000. ABI3 affects plastid differentiation in dark-grown Arabidopsis seedlings. *The Plant Cell* **12,** 35–52.

Shen Q, Ho THD. 1995. Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. *The Plant Cell* **7,** 295–307.

Shen Q, Uknes S, Ho THD. 1993. Hormone response complex in a novel abscisic acid and cycloheximide inducible barley gene. *Journal of Biological Chemistry* **268,** 23652–23660.

Shen Q, Zhang P, Ho THD. 1996. Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. *The Plant Cell* **8,** 1107–1119.

Skriver K, Olsen FL, Rogers JC, Mundy J. 1991. Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proceedings of the National Academy of Sciences, USA* **88,** 7266–7270.

Staub JM, Deng XW. 1996. Light signal transduction in plants. *Photochemistry and Photobiology* **64,** 897–905.

Steber CM, McCourt P. 2001. A role for brassinosteroids in germination in Arabidopsis. *Plant Physiology* **125,** 763–769.

Sullivan JA, Gray JC. 1999. Plastid translation is required for the expression of nuclear photosynthesis genes in the dark and in roots of the pea *lip1* mutant. *The Plant Cell* **11,** 901–910.

Sullivan JA, Gray JC. 2002. Multiple plastid signals regulate the expression of the pea plastocyanin gene in pea and transgenic tobacco plants. *The Plant Journal* **32,** 763–774.

Susek R, Ausubel FM, Chory J. 1993. Signal transduction mutants of Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. *Cell* **74,** 787–799.

Tai HH, Pelletier C, Beardmore T. 2004. Total RNA isolation from *Picea mariana* dry seed. *Plant Molecular Biology Reporter* **22,** 93a–93e.

Thompson WF, White MJ. 1991. Physiological and molecular studies of light-regulated nuclear genes in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **42,** 423–466.

Vasil V, Marcotte WR, Rosenkrans L, Cocciolone SM, Vasil IK, Quatrano RS, McCarty DR. 1995. Overlap of Viviparous1 (VP1) and abscisic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. *The Plant Cell* **7,** 1511–1518.

Weatherwax SC, Ong MS, Degenhardt J, Bray EA, Tobin EM. 1996. The interaction of light and abscisic acid in the regulation of plant gene expression. *Plant Physiology* **111,** 363–370.

Wei N, Deng XW. 1996. The role of the *COP*/*DET*/*FUS* genes in light control of Arabidopsis seedling development. *Plant Physiology* **112,** 871–878.

Williams ME, Foster R, Chua NH. 1992. Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. *The Plant Cell* **4,** 485–496.

Zou J, Brokx SJ, Taylor DC. 1996. Cloning of a cDNA encoding the 21.2 kDa oleosin isoform from *Arabidopsis thaliana* and a study of its expression in a mutant defective in diacylglycerol acyltransferase activity. *Plant Molecular Biology* **31,** 429–433.