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., 2006b).

Photomorphogenesis is facilitated through several photoreceptors recognizing different regions of the light...
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., 2006a) 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 LHCBI (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 aprF (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 Plains, 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 period, 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, Bromad, UK) and fine vermiculite (William Sinclair Horticulture, Lincoln, UK). The soil mixture was watered with 0.2 g l⁻¹ Interceptor 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
seeds were 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 (\( \mu g \) of chlorophyll g\(^{-1}\) of seedling fresh weight).

Transmission electron microscopy

The process was performed as described in Perachia 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 CuCl\(_2\). 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 tetroxide for 1 h, rinsed three times in deionized water, and stained in 2\% (w/v) uranyl acetate for 1 h.

Transmission electron microscopy

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 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 \( \mu g \)) was separated by electrophoresis on formaldehyde–1.2% (w/v) agarose gels and then transferred to sheets of nitrocellulose 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 [\( \alpha \] -\( ^{32} \)P]dATP (40 \( \mu Ci/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\( ^{\text{cin}} \) (At5g09810) was used in all statistical analysis relative to the upstream region of other (unselected) genes.

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 \( \mu l \)) was incubated for 45 min with 100 \( \mu l \) of \( (\text{dL-cis,trans-[G-3H]}\) ABA (Amersham Biosciences) and 100 \( \mu l \) of MAC 252 monoclonal antibody against \( (S)\text{-cis,trans-ABA} \) (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 \( \mu l \) 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).

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](image-url). 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.
Most (11 out of 13) of the lower amounts of transcripts showed at least 3-fold lower transcript levels in eight genes encoding oleosin proteins, all except one gene showed very low transcript amounts in proteins, including both 12S globulins and 2S albumins, gene probes representing genes encoding seed storage proteins, oleosins, and late embryogenesis abundant seed maturation, including genes encoding seed storage proteins, oleosins, and late embryogenesis abundant seed maturation (8–11 DAF), early embryogenesis [1–5 days after flowering (DAF)], maturation (8–11 DAF), late embryogenesis (17–21 DAF), 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 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),
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
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 \textit{ged1} was observed in the amounts of transcripts of \textit{CRA1}, \textit{OLEO2}, and \textit{LEA76} during any of the stages of seed development. Hence, the seedling phenotype of \textit{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 \textit{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 \textit{Ws} and \textit{ged1} seedlings was subjected to RNA gel-blot analysis. Probes for \textit{RBCS}, \textit{LHCBI}, \textit{HEM}, \textit{CRA1}, \textit{OLEO2}, and \textit{LEA76} during any of the stages of seed development. Hence, the seedling phenotype of \textit{ged1} was unlikely to be a consequence of decreased expression during embryogenesis and seed maturation.

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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 \textit{Ws} and \textit{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 \textit{Ws} and \textit{ged1} using Concert Plant RNA Reagent, and 15 μg of the total RNA was subjected to RNA gel-blot analysis using ³²P-labelled probes from genes encoding CRA1, OLEO2, and LEA76. Ethidium bromide-stained rRNAs are shown as a loading control.
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 LHCBI and RBCS transcripts in early development of dark-grown Arabidopsis seedlings reported by Brusslan and Tobin (1992).

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, LHCBI, 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, LHCBI, 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;
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; ACGCGGTGTCCTC), 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
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-typeWs 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 ≥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 darkness), 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
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 \( \text{CRA1} \), \( \text{OLEO2} \), and \( \text{LEA76} \) were detected in 2- to 10-day-old \( \text{ged1} \) seedlings. The temporal patterns of transcript accumulation and decline in wild-type seedlings were not observed in \( \text{ged1} \) seedlings. The marked increase in seed protein gene transcripts observed in illuminated 10-day-old wild-type seedlings did not occur in \( \text{ged1} \) 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 \( \text{ged1} \) 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 \( \text{ged1} \) showed that they shared ACGT-containing elements, similar to ABREs (Guiltnan 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

| Sequence     | Observed in the selected genes (%) | Observed in unselected genes (%) | Random rate (%) | P-value | Random rate (%) | P-value |
|--------------|------------------------------------|----------------------------------|-----------------|---------|-----------------|---------|
| ACGTGT       | 109/279 (39.1)                     | 16.6                             | 15.0            | 3.8×10^{-15} | 109/279 (39.1) | 16.6                             | 15.0            | 3.8×10^{-15} |
| CACGTCG      | 105/279 (37.6)                     | 15.7                             | 8.0             | 7.6×10^{-15} | 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} | 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} | 64/279 (22.9) | 6.8                              | 2.7             | 4.7×10^{-13} |
| ACACGTG      | 66/279 (23.7)                      | 5.3                              | 2.7             | 1.6×10^{-12} | 66/279 (23.7) | 5.3                              | 2.7             | 1.6×10^{-12} |
| CGTGTC       | 77/279 (27.6)                      | 10.1                             | 7.9             | 2.1×10^{-12} | 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} | 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 (Pennell 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 F2 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 gedl 7-day-old seedlings relative to wild-type Ws seedlings.

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

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

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

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

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

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