Atypical E2F activity coordinates PHR1 photolyase gene transcription with endoreduplication onset

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Because of their sessile life style, plants have evolved the ability to adjust to environmentally harsh conditions. An important aspect of stress adaptation involves the reprogramming of the cell cycle to ensure optimal growth. The atypical E2F transcription factor DP-E2F-like 1 (E2Fe/DEL1) had been found previously to be an important regulator of the endocycle onset. Here, a novel role for E2Fe/DEL1 was identified as a transcriptional repressor of the type-II cyclobutane pyrimidine dimer-photolyase DNA repair gene *PHR1*. Upon ultraviolet-B (UV-B) treatment, plants knocked out for *E2Fe/DEL1* had improved DNA repair abilities when compared with control plants, whereas those overexpressing it performed less well. Better DNA repair allowed *E2Fe/DEL1* knockout plants to resume endoreduplication faster than control plants, contributing in this manner to UV-B radiation resistance by compensating the stress-induced reduction in cell number by ploidy-dependent cell growth. As *E2Fe/DEL1* levels decreased upon UV-B treatment, we hypothesize that the coordinated transcriptional induction of *PHR1* with the endoreduplication onset contributes to the adaptation of plants exposed to UV-B stress.

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Introduction

Ultraviolet-B (UV-B) radiation (280–315 nm) is a high-energy component of sunlight that is potentially damaging to life on

earth. Plants deal with radiation by avoidance and repair. Avoidance mechanisms include the accumulation of shielding compounds synthesized upon perceiving UV-B, such as UV-absorbing secondary metabolites and free radical scavengers (Jansen *et al*, 1998), among which flavonoids are some of the most common found to accumulate after UV-B irradiation (Stapleton and Walbot, 1994; Rozema *et al*, 2002). Correspondingly, mutant *Arabidopsis thaliana* plants with increased levels of flavonoids and other phenolic compounds exhibit tolerance to otherwise lethal UV-B levels (Bieza and Lois, 2001). When shielding is insufficient, harmful compounds, such as free radicals, are produced, potentially affecting proteins, transcription, and replication. The most frequent UV-specific damages caused to DNA molecules are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) cross-links, representing ~75% and 25% of the damage generated by UV radiation, respectively (Britt, 1996). CPDs and 6-4PPs prevent the DNA molecule to be replicated or transcribed (Sancar, 1994); hence, their quick elimination is crucial to permit growth. CPDs are specifically removed by photoreactivation, performed by enzymes called photolyases, which use natural sunlight to repair the UV-B triggered DNA damage (Ahmad *et al*, 1997; Waterworth *et al*, 2002). The photolyase photoreactivating enzyme/UV-resistant 2 (PHR1/UVR2) contains two chromophores, of which one is a flavin FADH[−] cofactor that acts as electron donor and directly reverts CPDs in an error-free manner and the other one confers the specificity (Britt, 1999). Depletion of photolyase activity in *Arabidopsis* leads to incomplete CPD photoreactivation and defects of root growth after irradiation (Jiang *et al*, 1997; Landry *et al*, 1997; Waterworth *et al*, 2002). Conversely, an increase in CPD photolyase activity can minimize the growth-inhibitory effects of UV-B in rice (*Oryza sativa*) and *Arabidopsis* (Hidema *et al*, 2007; Kaiser *et al*, 2009). 6-4PPs are removed by another photolyase enzyme, namely UVR3 (Jiang *et al*, 1997).

PHR1 expression in *Arabidopsis* is controlled by light (Waterworth *et al*, 2002). The pathway through which darkness inhibits photolyase expression and white light triggers it, has been unraveled recently and involves an interplay of different photomorphogenic regulators, including HY5, HYH, DET1, and COP1 (Castells *et al*, 2010). Interestingly, in contrast to other DNA repair genes tested, *PHR1* expression in rice is not restricted to dividing cells, but can be detected additionally in maturing tissues, suggesting a role for the enzyme in the protection of post-mitotic cells (Kimura *et al*, 2004).

Cell division is a primordial aspect of plant life and needs to be tightly regulated to allow plant growth and development at the right moment. The oscillating activity of cyclins and cyclin-dependent kinases (CDKs) is responsible for driving cells alternately through DNA replication and mitosis (Inzé and De Veylder, 2006). Besides this classical cell cycle, some cells might undergo an alternative cell cycle in which mitosis and cytokinesis are skipped, but replication

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continues, resulting into an increase of the cellular DNA ploidy level (Lee *et al*, 2009). This phenomenon, called endoreduplication or endopolyploidy, is widespread among plants (Galbraith *et al*, 1991), although its physiological relevance is only poorly understood. Various beneficial roles have been proposed for the endoreduplication process, such as compensation for a small genome to support cellular activities (Nagl, 1976), urge for cell expansion (Melaragno *et al*, 1993), and support of high transcription rates (Galitski *et al*, 1999) and of development of specific cell structures, such as trichomes (Hülkamp *et al*, 1994; Bramsiepe *et al*, 2010). Under environmental harsh conditions, endopolyploid plants are proposed to develop faster than plants without endopolyploidy because they combine the advantages of both small and large genome species. A small genome allows cells to duplicate quickly at a low-energy cost, while a large genome enables maintenance of large cells. These features can be valuable under conditions that limit cell division, but not cell elongation, such as cold climates (Barow and Meister, 2003). Another popular hypothesis is that the increase in gene copy number might buffer the loss of essential genes, as, for instance, by DNA damage through irradiation (Kondorosi and Kondorosi, 2004; Hase *et al*, 2006). Consistent with the latter hypothesis, a mutation in the *Arabidopsis* *UVI4* gene increases endoreduplication and confers UV-B tolerance in parallel (Hase *et al*, 2006).

The E2F/DP transcription factors have been reported as important regulators of the mitotic and endoreduplication cell cycle and are highly conserved in mammals and plants. *Arabidopsis* contains six E2F transcription factors, subdivided into typical and atypical E2Fs (Lammens *et al*, 2009). The typical E2Fs (E2Fa–E2Fc) possess one DNA binding and one dimerization domain, enabling the binding to the DP partner. The DP partner itself contains a second DNA-binding domain and is required for high-affinity binding to the promoters of E2F target genes, including DNA repair and replication genes (e.g. *CDC6*, *MCM3*, *ORC1*, and *PCNA*) (Vlieghe *et al*, 2003; Vandepoele *et al*, 2005). By contrast, atypical E2Fs (E2Fd–E2Ff, also known as DP-E2F-like [DEL] proteins) lack a dimerization domain, but hold a duplicated DNA-binding domain that allows them to bind to the same consensus *cis*-acting element as the typical E2Fs, namely TTTSSCGS (where S is a C or a G). Atypical E2Fs lack a clear transcriptional activation domain and are, therefore, thought to be transcriptional repressors (Lammens *et al*, 2009).

Previously, we have found that the *Arabidopsis* E2Fe/DEL1 protein represents an important regulator of the mitosis-to-endocycle transition (Vlieghe *et al*, 2005; Lammens *et al*, 2008). E2Fe/DEL1 represses the endocycle onset through transcriptional repression of the *CCS52A2* gene, a CDH1-related activator of the anaphase-promoting complex (APC) ubiquitin ligase. In the presence of E2Fe/DEL1, the APC activity is restrained, resulting in the accumulation of G2-M-specific cyclins that drive mitosis. By contrast, upon loss of E2Fe/DEL1, the APC in complex with *CCS52A2* destroys the mitotic CDK/cyclin complexes, stimulating the endoreduplication onset. Consequently, *E2Fe/DEL1* knockout (*E2Fe/DEL1*^{KO}) plants display increased DNA ploidy levels, whereas overexpressor lines (*E2Fe/DEL1*^{OE}) show reduced endoreduplication (Vlieghe *et al*, 2005). Here, we identify the *PHR1* gene as an additional new target gene of *E2Fe/DEL1*, connecting UV-B sensitivity with endocycle onset.

Results

E2Fe/DEL1 controls *PHR1* transcription

In a microarray experiment that compared the transcriptome of wild-type *Arabidopsis* plants with that of transgenic plants either overexpressing or knocked out for *E2Fe/DEL1*, the photolyase gene *PHR1* was identified as one of the genes of which the expression level was significantly modulated (Lammens *et al*, 2008). To confirm this result, we investigated the *PHR1* transcript level in control versus *E2Fe/DEL1*^{OE} and *E2Fe/DEL1*^{KO} leaves by quantitative real-time PCR analysis. The transcript levels of *PHR1* were ~2.9-fold higher and 4.0-fold lower in the *E2Fe/DEL1*^{KO} and *E2Fe/DEL1*^{OE} lines than those of the wild-type plants (Figure 1A). These data show that E2Fe/DEL1 operates as a transcriptional repressor of the *PHR1* gene. Consistently, the *PHR1* promoter holds a putative E2F-binding site (AAACCGGC) located 459 bp upstream of its translation start site. To assess whether E2Fe/DEL1 controls the *PHR1* transcription activity through direct binding to its promoter, a chromatin immunoprecipitation (ChIP) experiment was performed using an E2Fe/DEL1-specific antibody (Lammens *et al*, 2008). *E2Fe/DEL1*^{KO} plants were used as a negative control. ChIP with primers spanning the E2F-binding site clearly resulted in an enriched precipitation of the *PHR1* promoter in wild-type versus *E2Fe/DEL1*^{KO} samples (Figure 1B). Locus scanning by quantitative real-time PCR with eight primer pairs covering the first 1366 base pairs of the *PHR1* promoter showed a preferential association with the region comprised between –304 and –506 base pairs upstream of the start codon (Figure 1C), matching the position of the predicted E2F-binding site. According to these

Figure 1 Regulation of *PHR1* transcription by E2Fe/DEL1. (A) *PHR1* levels in the first leaf pair of 14-day-old seedlings. All values were normalized to the housekeeping gene *ACT2*. Measurements were made relative to Col-0 wild-type (WT) and are represented as mean ± s.d. ($n = 3$, P -value Student's t -test, * <0.01 ; ** <0.05). (B) ChIP analysis showing binding of E2Fe/DEL1 to the *PHR1* promoter. (C) ChIP scanning of the *PHR1* promoter showing the strongest association of E2Fe/DEL1 around the putative E2F *cis*-acting element. Numbers in the x-axis correspond to the start and stop position of the promoter fragment tested. Data show the fold enrichment, representing the ratio between primer fragment amplification in control and *E2Fe/DEL1*^{KO} plants.

results, *E2Fe/DEL1* probably operates as a direct transcriptional regulator of the *PHR1* gene.

***E2Fe/DEL1* levels correlate with recovery from UV-B radiation damage**

As transcript abundance does not always reflect enzyme activity, photolyase activity levels were measured in the different transgenic lines by irradiating plants with 116 kJ/m² UV-B (5 h treatment). After treatment, plants were either harvested immediately or after an additional 5-h white light treatment, allowing CPD repair. The analysis focused on leaf 5, because it expressed *E2Fe/DEL1* at the time the treatment was applied. In all three lines, the accumulation of CPDs was equal in plants harvested directly after the UV-B treatment, illustrating that modified *E2Fe/DEL1* levels do not provide any protective benefit (Figure 2). Five hours of recovery in white light allowed control plants to repair ~34% of the CPDs, in contrast, to 51% and only 16% in *E2Fe/DEL1*^{KO} and *E2Fe/DEL1*^{OE}, respectively. These data demonstrate that the changes in *PHR1* transcript due to knockout or overexpression of *E2Fe/DEL1* are accompanied by changes in the CPD repair rate. The amount of CPDs of non-treated plants did not differ significantly (Supplementary Figure S1), probably because under these conditions the *PHR1* levels are not limiting to repair the low level of CPDs that occur in the absence of UV-B radiation.

***E2Fe/DEL1*^{KO} plants display increased tolerance towards UV-B**

To test whether the observed changes in *PHR1* transcript levels and CPD repair correlated with a differential response of the transgenic plants towards the UV-B treatment, 18-day-old plants were transferred to a sun simulator where they were submitted to four different doses of UV-B, corresponding to 35 kJ/m² (3 h treatment), 59 kJ/m² (5 h), 82 kJ/m² (7 h), and 106 kJ/m² (9 h), and photographed 9 days after the UV-B irradiation. No phenotypic differences could be observed for the non-treated plants (Figure 3A). Increasing doses of UV-B caused macroscopic damage. Additionally,

Figure 2 Efficient CPD repair of *E2Fe/DEL1*^{KO} plants. CPD concentrations were measured by ELISA in the fifth leaves irradiated with 116 kJ/m² UV-B, immediately harvested after irradiation (dark) or after recovery for 5 h in white light (grey). The CPD amounts in the Col-0 wild-type (WT) plants harvested immediately after treatment was arbitrarily set to 1. The CPD repair was statistically better in *E2Fe/DEL1*^{KO} leaves than in control ($n = 3$, P -value Student's t -test, $* < 0.01$).

plants clearly showed a dose-dependent decrease in size and biomass (Figure 3A and B). However, the dose response depended clearly on the genotype. Whereas the control displayed a reduced size and biomass at 35 kJ/m² and clear damage at 59 kJ/m², *E2Fe/DEL1*^{KO} plants performed clearly better at these doses. By contrast, the *E2Fe/DEL1*^{OE} plants showed a stronger growth reduction than control plants at 35 kJ/m². These results illustrate an increased tolerance and sensitivity towards UV-B of *E2Fe/DEL1*^{KO} and *E2Fe/DEL1*^{OE} plants, respectively. A typical daily UV-B dose on a mid-latitude (48°N) summer day (at 500 m altitude) is 45 kJ/m² (own measurements), indicating that the *E2Fe/DEL1* plants display a differential response in the range of naturally occurring UV-B doses.

***E2Fe/DEL1*^{KO} plants overcome the inhibitory effects of UV-B radiation on cell cycle progression and endoreduplication**

Effects on organ size can be due either to a reduced cell number or cell size, resulting from an inhibition of cell division or cell expansion, respectively. To distinguish between these possibilities, we measured the leaf size, abaxial epidermal cell size, and cell number of control and *E2Fe/DEL1*^{KO} plants. Measurements were done on the fifth leaf that still divided at the moment of the UV-B treatment and, thus, expressed *E2Fe/DEL1* (Lammens *et al*, 2008). Control and *E2Fe/DEL1*^{KO} plants were treated with 35 kJ/m² (3-h treatment) of UV-B and harvested after 9 days. Leaf size, cell number, and cell size were comparable under untreated conditions in *E2Fe/DEL1*^{KO} and wild type. The leaf size decreased after UV-B treatment in both lines, but the effect was significantly (P -value Student's t -test < 0.05) less for *E2Fe/DEL1*^{KO}, reaching 65.9% of the area of the non-irradiated plants versus only 39.5% for wild-type leaves (Figure 4A). The abaxial epidermal cell number was reduced by 42.3% in the UV-B-treated *E2Fe/DEL1*^{KO} leaves versus 55.4% in the control plants (Figure 4B), illustrating that the cell division in the *E2Fe/DEL1*^{KO} plants was less affected. Average cell size was slightly, but significantly (P -value Student's t -test < 0.01) reduced in the control, but not in *E2Fe/DEL1*^{KO} (Figure 4C). Interestingly, after UV-B treatment, the cell size of *E2Fe/DEL1*^{KO} plants was slightly, but significantly, larger than that of wild-type plants (P -value Student's t -test < 0.01). As cell size is in part determined by the DNA content (Melaragno *et al*, 1993; Sugimoto-Shirasu and Roberts, 2003), DNA ploidy levels were measured through flow cytometry at a 3-day interval after irradiation. As reported previously, under control conditions, the endoreduplication index (EI) of *E2Fe/DEL1*^{KO} plants increased faster than that of wild-type plants as the leaf matured (Vlieghe *et al*, 2005; Lammens *et al*, 2008). UV-B treatment delayed the increase in EI in both lines, but 12 days after irradiation, the EI level of the *E2Fe/DEL1*^{KO} plants regained control levels, whereas it remained reduced in wild-type plants (Figure 4D). These data indicate that the absence of *E2Fe/DEL1* helps in overcoming the inhibitory effect of UV-B irradiation on the endocycle progression.

Ploidy-driven cell growth aids recovery of leaf growth after UV-B irradiation

To test whether the sustained endoreduplication of the *E2Fe/DEL1*^{KO} plants contributes to their observed UV-B tolerance,

Figure 3 Growth dose response of Col-0 wild-type (WT), *E2Fe/DEL1*^{KO}, and *E2Fe/DEL1*^{OE} plants towards UV-B. (A) Eighteen-day-old plants irradiated in a sun simulator with the indicated doses. Plants were photographed 9 days after treatment. (B) Fresh weight measurements of untreated and UV-B irradiated plants. Data are represented as mean $\pm$ s.e. ($n > 3$).

we measured the DNA ploidy level and size of individual pavement cells, both under control and UV-B treated (35 kJ/m²) conditions. As observed for the complete leaf, UV-B treatment resulted into a clear decrease in the relative abundance of pavement cells with 8C and 16C DNA content in control plants (Table I). By contrast, endoreduplication was stimulated in the *E2Fe/DEL1*^{KO} background, as exemplified by the increase in the 8C population. As reported before (Melaragno *et al*, 1993), within every DNA ploidy class a broad distribution in cell sizes was observed, reflecting ploidy-independent growth (Table I). However, for both genotypes, the DNA content of a cell and its average size correlated clearly, with 16C cells $\sim$ 5–10-fold larger than those with a 2C DNA content, indicating ploidy-dependent growth (Table I). To estimate the relative contribution of each DNA ploidy class to the final size of the leaf, the proportion of cells within a given DNA ploidy class was multiplied with the average cell size. In wild-type plants, the 8C and 16C cells accounted for $\sim$ 70% of the growth under non-stress conditions, but their contribution dropped to 50% after UV-B treatment (Table I). In contrast, the two highest DNA ploidy classes contributed for 64–70% to the *E2Fe/DEL1*^{KO} leaf size under both conditions, which can be attributed mainly to the

high proportion of 8–16C cells. Thus, *E2Fe/DEL1*^{KO} plants apparently triggered a better growth performance after UV-B treatment because of the presence of high-ploidy cells, which on average are larger than the cells with a low-ploidy level.

Endocycle-dependent leaf recovery after UV-B irradiation is linked with *E2Fe/DEL1* expression levels

As the *E2Fe/DEL1* gene is predominantly active in dividing tissues (Lammens *et al*, 2008), mainly leaves that are mitotically active would be expected to benefit from the absence of *E2Fe/DEL1* at the moment of the UV-B treatment. To test this hypothesis, we compared the recovery of a young leaf (leaf 5) with that of an older one (leaf 3). When the UV-B treatment was applied, the fifth leaf was still predominantly dividing, whereas the third became less mitotically active, as quantified by their difference in *E2Fe/DEL1* transcript levels (Figure 5A). When the reduction in leaf size was measured of irradiated versus untreated plants 9 days after the UV-B treatment, both the young and old *E2Fe/DEL1*^{KO} leaves performed better than those of the wild-type plants (Figure 5B), but the recovery was clearly more pronounced in the youngest leaf. Flow cytometric analysis indicated that the endocycle progression was clearly affected in both the young and

Figure 4 Leaf growth analysis of Col-0 wild-type (WT) and *E2Fe/DEL1^{KO}* plants under UV-B treatment. (A–C) Eighteen-day-old plants were not treated (white) or irradiated with 35 kJ/m² UV-B in a sun simulator (dark). The fifth leaves were harvested 9 days after irradiation for leaf size (A), epidermal cell number (B), and epidermal cell size (C). (D) Endoreduplication kinetics. Eighteen-day-old wild-type (black) and *E2Fe/DEL1^{KO}* (grey) plants were not treated (full lines) or irradiated with 35 kJ/m² UV-B (dashed lines). Data are represented as mean ± s.e. (*n* = 5).

Table 1 Correlation between the DNA ploidy level and cell size of Col-0 wild-type (WT) and *E2Fe/DEL1^{KO}* pavement cells under control (–UV-B) and UV-B-irradiated (+UV-B) growth conditions

Treatment and line	DNA ploidy class	Number of cells (% of total)	Average cell area in µm ² (range)	Contribution to total leaf size (%)
WT–UV-B (<i>n</i> = 332)	2C	85 (26%)	707 (127–2704)	6.4
	4C	115 (35%)	1867 (508–6346)	23.0
	8C	100 (30%)	4433 (899–13 354)	47.5
	16C	32 (10%)	6735 (6212–13 710)	23.1
WT + UV-B (<i>n</i> = 332)	2C	82 (24%)	849 (133–2715)	10.4
	4C	146 (44%)	1787 (237–4916)	39.1
	8C	91 (27%)	3037 (293–6039)	41.4
	16C	12 (4%)	4608 (1590–8457)	9.0
DEL1 ^{KO} –UV-B (<i>n</i> = 333)	2C	97 (29%)	1021 (218–2835)	9.2
	4C	103 (31%)	2740 (321–8221)	26.3
	8C	77 (23%)	3806 (1302–8556)	27.1
	16C	56 (17%)	7170 (3528–20 475)	37.4
DEL1 ^{KO} + UV-B (<i>n</i> = 332)	2C	65 (20%)	1014 (162–3348)	6.5
	4C	99 (30%)	2270 (343–7009)	22.0
	8C	106 (32%)	3641 (1339–7927)	37.7
	16C	62 (19%)	5596 (1996–11 789)	33.8

old *E2Fe/DEL1* leaves (Figure 5C and D). However, whereas the EI levels remained relatively low in the oldest leaf in the treated versus untreated plants during the first 9 days after irradiation, the EI increased steadily in the young leaf. These data indicate that the young leaves are more proficient in overcoming the inhibition of endoreduplication by UV-B irradiation.

PHR1 activity aids endoreduplication

The recovery of the endoreduplication process in *E2Fe/DEL1^{KO}* leaves suggested that the PHR1 activity might aid endocycle progression. To test this hypothesis, the DNA

ploidy levels of a control and a *PHR1* knockout (*PHR1^{KO}*) line were compared. Eighteen-day-old plants were irradiated with 34 kJ/m² UV-B and 9 days later, the fifth leaf was harvested for DNA ploidy analysis. In the absence of UV-B, the EI did not differ significantly in the wild-type and *PHR1^{KO}* lines (Figure 6). The UV-B treatment affected the DNA ploidy distribution only marginally in the control background (Figure 6A and B). By contrast, endoreduplication was severely inhibited in the *PHR1^{KO}* plants (Figure 6C–E), indicating that the *PHR1* expression is required to overcome the inhibitory effect of UV-B on the endocycle progression.

Figure 5 Leaf age-dependent recovery in growth and endoreduplication after UV-B irradiation. (A) *E2Fe/DEL1* transcript levels in the third (L3) and fifth (L5) leaf of Col-0 wild-type (WT) and *E2Fe/DEL1*^{KO} plants. (B) Eighteen-day-old plants irradiated with 59 kJ/m² UV-B. Radiation recovery is plotted as the percentage reduction in leaf size of the UV-B treated versus untreated plants. (C, D) *E2Fe/DEL1*^{KO} plants harvested at a 3-day interval to measure the endoreduplication kinetics in third (C) and fifth (D) untreated (grey) and irradiated (dark) leaves. Data are represented as mean $\pm$ s.e. ($n > 10$).

E2Fe/DEL1 transcripts are repressed by UV-B

Because of its observed link with *PHR1*, we tested whether *E2Fe/DEL1* transcription is controlled by UV-B radiation. Eighteen-day-old seedlings were irradiated with 35 kJ/m² UV-B and harvested immediately after the treatment (5 h after onset) for RNA extraction. Within this short treatment, *PHR1* levels increased approximately six-fold (Figure 7A). *PHR1* levels were higher in the *E2Fe/DEL1*^{KO} plants under both control and UV-B conditions, but its level of induction was identical to that observed in control plants, indicating that *E2Fe/DEL1* did not interfere with induction of *PHR1* by UV-B. In contrast, *E2Fe/DEL1* levels were strongly downregulated by UV-B treatment (Figure 7B).

Discussion

PHR1 is an *E2Fe/DEL1* target gene

UV-B is a component of the sunlight in the biosphere. It can potentially damage plant membranes, proteins, and DNA. A recent sequence comparison between the genome of a reference strain with that of its descendants indicated that UV-B radiation could be in part responsible for the overall mutation rate of 7×10^{-9} base substitutions per site per generation (Ossowski *et al*, 2010). Plants try to overcome the UV-B-induced DNA damage through photoreactivation by photolyases (Britt, 1999). Here, we identified *E2Fe/DEL1* as a transcriptional repressor of *PHR1* that controls the photolyase activity level of cells. *E2Fe/DEL1* appears not to control the induction of *PHR1* by UV-B, but rather its basal expression level. Previously, we have found that *E2Fe/DEL1* operates as a repressor of the endocycle onset through transcriptional repression of the APC activator gene

CCS52A2 (Vlieghe *et al*, 2005; Lammens *et al*, 2008). As demonstrated, UV-B inhibited both the mitotic cell cycle and endocycle, indicating that CPDs interfere with the polymerases that duplicate the genome in mitotically dividing and endocycling cells. When compared with those of control plants, both processes were significantly less inhibited in the *E2Fe/DEL1*^{KO} plants. Likely, the higher *PHR1* levels under UV-B conditions contributed to an accelerated removal of CPDs, probably contributing to a more efficient untangling of damaged DNA, and thus resulting in a fast resumption of DNA replication. Interestingly, *E2Fe/DEL1* levels were quickly downregulated upon UV-B treatment, suggesting a role in the DNA damage response (DDR). Mammalian atypical E2Fs (*E2F7* and *E2F8*) have also been linked with DDR, steering the decision between cell survival and apoptosis of a DNA-damaged cell. Low *E2F1* levels stimulate DNA repair, whereas high *E2F1* activity triggers the transcription of genes driving the apoptotic program (Lin *et al*, 2001; Stevens and La Thangue, 2003; Stevens *et al*, 2003). *E2F1* appears to be a direct *E2F7/E2F8* target gene and in the absence of both atypical E2Fs, cells massively undergo apoptosis (Li *et al*, 2008; Zalmas *et al*, 2008). As discussed previously (Cools and De Veylder, 2009), plants have no need for an apoptotic program to eliminate severely damaged cells due to the lack of cell migration. As plant germ cells originate from somatic lineages, transmission of damaged DNA might be prevented by stimulating cells to enter the differentiation pathway, safeguarding the progeny from DNA mutations. Recently, endoreduplication has been demonstrated to be an essential step in the differentiation of cells (Bramsiepe *et al*, 2010). Therefore, downregulation of *E2Fe/DEL1* might be an initial trigger to eliminate potentially harmful dividing

Figure 6 Requirement of PHR1 activity for efficient endoreduplication. (A–D) Representative DNA ploidy distributions of leaf 5 of 25-day-old *Ler* wild-type (WT) (A, B) and *PHR1*^{KO} (C, D) plants, either untreated (A, C) or irradiated with 34 kJ/m² UV-B 15 days after sowing (B, D). (E) Endoreduplication index. Data are represented as mean $\pm$ s.e. ($n = 10$).

cells by promoting their differentiation through upregulation of *CCS52A2* transcription, whereas the accompanying increase in *PHR1* levels might ensure that endocycle progression can occur, contributing to plant growth (see below).

Coordinated PHR1 expression and endocycle onset aid leaf growth

Endoreduplication has been suggested to aid in the protection against UV-B radiation, as indicated by the increased UV-B tolerance of the *uvi4* mutant that displays an increased DNA ploidy level (Hase *et al*, 2006). The presence of multiple copies of crucial genes could reduce the risk of losing essential genes by gene mutation or help repair of damaged copies by homologous recombination with an intact copy as template (Kondorosi and Kondorosi, 2004). However, the latter seems to be unlikely, because alignment and cohesion of the replicated sister chromatids are rapidly lost in endoreduplicating cells (Schubert *et al*, 2006). Moreover, double-stranded repair by homologous recombination is strongly inhibited in differentiated tissues (Boyko *et al*, 2006).

Figure 7 Effect of UV-B on *E2Fe/DEL1* and *PHR1* transcript levels. (A) *PHR1* transcript levels in 18-day-old Col-0 wild-type (WT) and *E2Fe/DEL1*^{KO} control (white) versus UV-B-irradiated seedlings (59 kJ/m²) (black). Plants were harvested for RNA extraction immediately after treatment. (B) *E2Fe/DEL1* transcript levels in Col-0 wild-type (WT) plants 14 days after sowing of whole seedlings either untreated (–UV) or treated with 35 kJ/m² of UV-B (+UV). Plants were harvested immediately after treatment. Data are represented as mean $\pm$ s.e. ($n = 3$).

Alternatively, endoreduplication might sustain growth of tissues by supporting cell expansion under conditions that prevent cell proliferation (Barow and Meister, 2003). The size of a leaf is determined by both cell proliferation and cell expansion. Under our experimental conditions, UV-B treatment resulted in a decrease of both cell number and the average cell size. The small reduction in leaf size of the mutant *E2Fe/DEL1*^{KO} plants upon radiation correlated with an elevated percentage of high-ploidy cells (8C and 16C), which can be explained by a fast resumption of the endoreduplication process after UV-B treatment due to a high basal *PHR1* level. Correspondingly, the endocycle progression was severely inhibited in *PHR1*-deficient plants under UV-B. As high-ploidy cells are on average larger than low-ploidy cells (2C and 4C), we speculate that *E2Fe/DEL1*^{KO} plants use the growth potential stored in their polyploid cells to compensate for the reduction in cell number. Efficient photorepair for the endocycle progression to aid growth might explain the need to coordinate the transcriptional control of *PHR1* and *CCS52A2* by *E2Fe/DEL1*, because coupling of DNA repair with replication gives the possibility of ploidy-driven cell growth to compensate for the UV-B-caused reduction in cell number. In this manner, a maximum leaf surface can be obtained after UV-B stress, allowing maximum photosynthesis to ensure sufficient energy to produce offspring.

Materials and methods

Plant material and growth conditions

All *A. thaliana* (L.) Heyhn. plants were of the Columbia-0 (Col-0) or Landsberg (*Ler*) ecotype. The transgenic plants *E2Fe/DEL1*^{KO} (Col-0) and *E2Fe/DEL1*^{OE} (Col-0) had been described previously (Vlieghe *et al*, 2005). The *phr1* line (*Ler*) was obtained from the European Arabidopsis Stock Center (catalogue number N8325). Plants were grown under control conditions of a 16-h photoperiod under white light exposure (TL-D 36W840 tubes; Philips, Eindhoven, The Netherlands). For UV-B treatment, Q-Panel UV-B-313EL tubes were added. A 95- μ m thick cellulose acetate film (Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany) placed between the light source and the plants prevented any UV-C radiation to reach the plants. Light and UV-B intensities were measured with a SpectroSense2+ device (SKYE, Powys, UK) and equaled 70 μ mol/m²/s and 1.9 W/m², respectively. For sun simulator treatments, the photosynthetically active radiation (PAR; 400–700 nm) was 172 μ mol/m²/s ($\pm 5\%$) and the UV-B irradiance 3.27 W/m² ($\pm 5\%$) under soda lime 4 mm Sanalux glass sheets

(SCHOTT DESAG AG, Grünplan, Germany). The climate parameters were adjusted to 75% relative humidity and 18°C temperature. Spectral irradiance was measured from 250 to 850 nm, with a double-monochromator TDM300 (Bentham, Reading, UK) in steps of 1 and 2 nm in the UV-B and visible range, respectively. During exposure, PAR, UV-A, and UV-B irradiances were continuously monitored. For more detailed information on the sun simulator treatments at the Helmholtz Zentrum München, see Döhring *et al* (1996) and Thiel *et al* (1996).

Flow cytometry analysis

Plant material was chopped in 200 µl of Cystain UV Precise P Nuclei extraction buffer (Partec, Münster, Germany), supplemented with 800 µl of staining buffer. The mix was filtered through a 50-µm green filter and read through the Cyflow MB flow cytometer (Partec). The nuclei were analysed with the FloMax software. The EI was calculated from the percentage values of each ploidy class with the formula: $EI = [(0 \times \%2C) + (1 \times \%4C) + (2 \times \%8C) + (3 \times \%16C) + (4 \times \%32C)]/100$.

CPD measurements

Two-week-old plants were submitted to UV-B radiation, then left to recover for 5 h in white light, and harvested before or after the recovery. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), titrated, and processed with the protocol for MBL D194-1 anti-CPD monoclonal antibody and ECL anti-mouse IgG horseradish peroxidase-linked whole antibody from sheep.

Real-time quantitative PCR analysis

Plants were grown on half-strength Murashige and Skoog medium, 0.8% agar at 20°C and 16 h photoperiod. RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized with the SuperScript Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). Quantitative PCR ran with a LightCycler (Roche) with the SYBR Safe enzyme reaction mix (Roche). The primers were forward *PHR1*, CTCGTTGGAGCAGTTAGAGAAGG; reverse *PHR1*, TGAACGC CACATATAGACCACATG; forward *DEL1*, TCTTCTCAGCCTCACTCTA GTAGC; and reverse *DEL1*, GCCGCCTCACTTTAGTTCCG. Actin was used as a control with forward and reverse primers GGCTCCTCTTA ACCCAAAGGC and CACACCATCACCAGAATCCAGC, respectively.

Chromatin immunoprecipitations

Eight days after sowing, whole seedlings were grown on half-strength Murashige and Skoog medium and 0.8% agar for ChIP as described (Bowler *et al*, 2004). Actin was used as a normalization gene with the following primers: forward, GGCTCCTCTTAACCCAA AGGC; reverse, CACACCATCACCAGAATCCAGC. The primers used to scan the *PHR1* promoter were CATTACGAAGGAGGAGAGACGG and GCAATCACAGTCTGACACGTGTTG (0–215), CAACACGTGTCAG ACTGTGATTGC and CCCGAACATCTATACACATTACAC (191–324), GTGTAATGTGTATAGATGTTCCGGG and GTTAGTTACGCTGTTAGGAG AGC (304–506), GCTCTCCTAACAG-CGTAACCTAAC and CTCTTGAA ATGGTCGTTTATACCTCAAC (487–746), GTTGAGGTATAAACGAC CATTTCAGAGC and CTGTTAGGACATTTGGACCATTAATGC (682–836), GCATTATGGTCCAAAATGCTCAACAG and GAGTAAACCCTAGATA AGCAGATC (809–1049), GATCGTGCTTATCTAGGGTTTACTC and

GAAAGAGTGATTATCATTCCTCATGATATG (1025–1164), CATATCAT GAGGAATGATAATCACTCTTTC and CATACGCTCAGCTAGTCGTT TG (1135–1366).

Epidermal peels

The protocol for epidermal peels was adapted from Melaragno *et al* (1993). Leaf 5 of 5-week-old plants were fixed in a solution containing $\frac{3}{4}$ of 95% ethanol and $\frac{1}{4}$ glacial acetic acid for 2 h at room temperature, stored in 70% ethanol at 4°C. Fixed tissue was soaked in water. Leaves were placed with the abaxial epidermis side down on a glass slide and held in place with forceps, while the tissue was removed from the adaxial epidermis with another pair of forceps. A drop of 4',6-diamino-2-phenylindole at a concentration of 0.005 mg/ml in McIlvaine's buffer, pH 4.1 (60 ml 0.1 M citric acid + 40 ml 0.2 M Na₂HPO₄), was placed on the epidermal peel. Peels were observed under a $\times 20$ objective on an Axioskop equipped with an Axiocam CCD camera (Zeiss). Images were obtained with the Axiovision software and were analysed with the public domain image analysis program ImageJ (version 1.40; <http://rsb.info.nih.gov/ij/>). Relative fluorescence units were measured as integrated density, which are the product of the area and the average fluorescence of the selected nucleus. Ten guard cell nuclei were used as a 2C control for each image. The growth contribution of each ploidy level to the final leaf surface (or LGC) was calculated according to the following formula:

$$LGC_i = \frac{p_i \times a_i}{\sum_{2C} p_i \times a_i}$$

where n is the maximum observed DNA ploidy classes in which i can take the value 2, 4, 8, 16, ..., p is the proportion of nuclei in a given ploidy class, and a is the average area associated to a given ploidy level.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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