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E2Fb and E2Fa transcription factors independently regulate the DNA damage response after ultraviolet B exposure in Arabidopsis

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SUMMARY

Ultraviolet (UV)B radiation affects plant growth inhibiting cell proliferation. This inhibition is in part controlled by the activity of transcription factors from the E2F family. In particular, the participation of E2Fc and E2Fe in UV-B responses in Arabidopsis plants was previously reported. However, the E2Fa and E2Fb contribution to these processes has still not been investigated. Thus, in this work, we provide evidence that, in Arabidopsis, both E2Fa and E2Fb control leaf size under UV-B conditions without participating in the repair of cyclobutane pyrimidine dimers in the DNA. Nevertheless, in UV-B-exposed seedlings, E2Fa, but not E2Fb, regulates primary root elongation, cell proliferation, and programmed cell death in the meristematic zone. Using *e2fa* mutants that overexpress *E2Fb*, we showed that the role of E2Fa in the roots could not be replaced by E2Fb. Finally, our results show that E2Fa and E2Fb differentially regulate the expression of genes that activate the DNA damage response and cell cycle progression, both under conditions without UV-B and after exposure. Overall, we showed that both E2Fa and E2Fb have different and non-redundant roles in developmental and DNA damage responses in Arabidopsis plants exposed to UV-B.

Keywords: cell proliferation, DNA damage response, E2F transcription factors, plant growth, UV-B radiation.

INTRODUCTION

Plants are naturally exposed to solar radiation, which is not only necessary for photosynthesis but also modulates their developmental programs. Although ultraviolet-B (UV-B) radiation, which comprises wavelengths from 280 to 315 nm, represents only a small proportion of the solar radiation that reaches the Earth, it significantly affects plant growth. In particular, UV-B exposure significantly affects plant growth, inducing a reduction of the leaf area and inhibition of primary root elongation (Casadevall et al., 2013; Fierro et al., 2015; Fina et al., 2017; Gómez et al., 2019; Wargent et al., 2009a, 2009b). In both leaves and roots, growth inhibition after UV-B exposure is because of a decrease in cell proliferation and/or in cell expansion; which may be affected differently by experimental conditions (Gómez et al., 2019; Hectors et al., 2010; Maulión et al., 2019). In particular, UV-B levels that produce DNA damage usually inhibit cell proliferation, whereas UV-B at lower doses and/or chronic irradiation can both inhibit cell proliferation and affect cell expansion (Dotto and Casati, 2017).

Previously, we showed that UV-B radiation, at intensities that produce DNA damage, inhibits cell proliferation, and this process is regulated by the E2Fc transcription factor (TF) in *Arabidopsis thaliana* plants (Gómez et al., 2019). E2Fc controls leaf size and primary root elongation under conditions of elevated UV-B radiation regulating DNA damage responses (DDR; Gómez et al., 2019). *E2Fc*-deficient plants had decreased programmed cell death (PCD) after irradiation with UV-B, and altered expression of *SOG1* and *ATR*, which encode proteins that regulate the DDR. E2Fc is one of the six E2F TFs (E2Fa-f) in Arabidopsis. While E2Fa, E2Fb, and E2Fc are typical E2Fs and are needed to heterodimerize with either two of the DP interaction proteins (DPa and DPb) to be functional; E2Fd/DEL2, E2Fe/DEL1, and E2Ff/DEL3 act independently of DP proteins and are known as atypical E2F/DEL TFs (Ramirez-Parra and Gutierrez, 2007). The different E2F TFs regulate the expression of

genes that control cell cycle progression and DNA replication (Naouar et al., 2009; Ramirez-Parra et al., 2003; Ramirez-Parra and Gutierrez, 2007; Vandepoele et al., 2005).

E2F activity is controlled by an evolutionary conserved repressor protein, retinoblastoma (Rb) (van den Heuvel and Dyson, 2008). When Rb is in an unphosphorylated state, it bounds to E2Fs, so they are not able to regulate gene expression; however, when Rb is phosphorylated by the CDKA/CYCD complex, E2Fs are released and drive the cell cycle by regulating the expression of cell cycle genes (Boniotti and Gutierrez, 2001; Magyar et al., 2012). In *A. thaliana*, a single gene encodes for RETINOBLATOMA RELATED (RBR), and this protein acts by binding the E2Fa–c TFs (De Veylder et al., 2007). Of the three typical E2F TFs, E2Fa and E2Fb are generally considered as transcriptional activators, while E2Fc usually acts as a repressor (Magyar et al., 2016).

E2Fa and E2Fb seemed to act redundantly during cell proliferation, as no viable plants could be recovered when some null mutants were combined (Li et al., 2017). However, a viable double *e2fab* mutant was isolated when particular loss-of function mutant alleles for *E2Fa* (*e2fa-2*) and *E2Fb* (*e2fb-1*) were combined, each mutant expressed proteins lacking the C-terminal transactivation domains, suggesting that these domains are dispensable for plant growth and development (Heyman et al., 2011). Using this *e2fab* mutant, it was then possible to analyze the specific roles of E2Fa and E2Fb in developing Arabidopsis seeds and embryos (Heyman et al., 2011; Leviczky et al., 2019). In fully developed embryos of the double mutant, cell number was not significantly affected, suggesting that the function of E2Fa and E2Fb is not required for embryonic cell proliferation. However, the expression of important genes during seed maturation such as *LEC1/2*, *ABI3*, and *FUS3* was increased in the double mutant embryos, suggesting that at least one of the two TFs could have a role as a repressor, restricting seed maturation. Moreover, results showed that during leaf development, E2Fb interacting with RBR, but not E2Fa, regulates cell proliferation, the exit to differentiation, and the establishment of quiescence, demonstrating that E2Fa and E2Fb have different roles regulating plant growth and development (Leviczky et al., 2019).

E2Fc and E2Fb are transcriptional regulators of the atypical *E2Fe/DEL1* TF under light conditions (Berckmans et al., 2011). E2Fb and E2Fc antagonistically control *E2Fe* transcript levels, competing for the same E2F-binding site. While E2Fb activates *E2Fe* expression, E2Fc represses it (Berckmans et al., 2011). Interestingly, E2Fe/DEL1 represses the transcription of a gene encoding a cyclobutane pyrimidine dimer (CPD) photolyase in Arabidopsis, *PHR1/UVR2*, and *E2Fe*-deficient plants accumulated less CPDs after UV-B exposure than wild-type (WT) plants (Radziejwoski et al., 2011). *e2fe/del1* mutants showed a premature onset of endoreduplication; and after UV-B exposure, they resumed endoreduplication faster than WT plants (Lammens et al., 2008; Radziejwoski et al., 2011; Vlieghe et al., 2005). Consequently, leaves from *E2Fe*-deficient plants showed a lower inhibition of growth after UV-B exposure, and they had larger cells with higher ploidy levels (Radziejwoski et al., 2011). E2Fc also regulates plant growth under UV-B conditions; however, the role of E2Fe in UV-B responses is independent of its regulation by E2Fc (Gómez et al., 2019). On the contrary, E2Fc is required for the regulation of cell proliferation under UV-B conditions mediated by the microRNA miR396, which also modulates leaf growth after exposure (Casadevall et al., 2013; Fina et al., 2017; Gómez et al., 2019). In addition, E2Fa was demonstrated to play roles maintaining genome integrity and viability in meristematic cells (Horvath et al., 2017). When DNA damage was produced with different genotoxic compounds, E2Fa together with RBR were recruited to the sites in the DNA where the damage occurred. Interestingly, E2Fa is also a transcription regulator of *BRCA1*, which encodes a DNA repair protein that participates in the DDR.

As described above, E2Fb is a light-dependent activator of E2Fe and regulates cell proliferation; however, its role in plant responses to UV-B radiation has not been investigated. Thus, in this work, we expanded our knowledge on the pathways regulated by E2F TFs during the DDR after UV-B exposure in Arabidopsis plants, and we analyzed a possible redundancy on the participation of E2Fa and E2Fb in this regulation. We investigated the role of both TFs in the regulation of leaf and primary root size after UV-B exposure. We here provide evidence that, while both E2Fa and E2Fb regulate leaf size under UV-B conditions, only E2Fa participates in the inhibition of primary root elongation in UV-B exposed seedlings. Moreover, our results show that while E2Fa regulates DDRs in the primary root, including cell death and cell proliferation, E2Fb does not participate in UV-B responses in this organ. *e2fab* double mutants showed a similar root phenotype after UV-B exposure as that of *e2fa* single mutant, validating the role of E2Fa in the DDR in the root. Interestingly, overexpression of *E2Fb* in an *e2fa* mutant background could not restore the WT root phenotype under UV-B conditions, suggesting that these TFs are not redundant during this process. Finally, we showed that E2Fa and E2Fb differentially regulate the expression of genes that activate the DDR and regulate cell cycle progression. Furthermore, the results showed that both E2Fa and E2Fb participate in plant responses after UV-B, and their roles are complementary and not redundant.

RESULTS

E2Fa and E2Fb regulate leaf growth after UV-B exposure

UV-B radiation at intensities that produce DNA damage inhibits leaf growth by affecting cell proliferation

(Casadevall et al., 2013; Fina et al., 2017; Gómez et al., 2019). Both E2Fc and E2Fe regulate cell proliferation in the leaves of UV-B-irradiated Arabidopsis plants (Gómez et al., 2019; Radziejwoski et al., 2011). *E2Fe* is negatively regulated by E2Fc; but its transcription is activated by E2Fb (Berckmans et al., 2011); thus, we investigated if E2Fb could also control leaf growth after irradiation with UV-B in Arabidopsis plants. The effect of UV-B in rosette and leaf growth was analyzed using two *e2fb Arabidopsis* T-DNA insertion mutants (*e2fb-1* and *e2fb-2*). When grown in the growth chamber in the absence of UV-B, *e2fb-1* and *e2fb-2* plants looked similar to WT Col-0 plants (Figure 1a). WT and mutant plants were irradiated with a single UV-B treatment for 4 h 12 days after stratification, when leaf no. 5 was emerging and had mostly proliferating cells (Casadevall et al., 2013). Two days after the end of the treatment, the rosette area was smaller in both WT and *e2fb* mutants than in control plants that were not UV-B-irradiated, and this persisted after the treatment (Figure 1a,b; Figure S1a). Nevertheless, UV-B-irradiated *e2fb* plants were bigger than

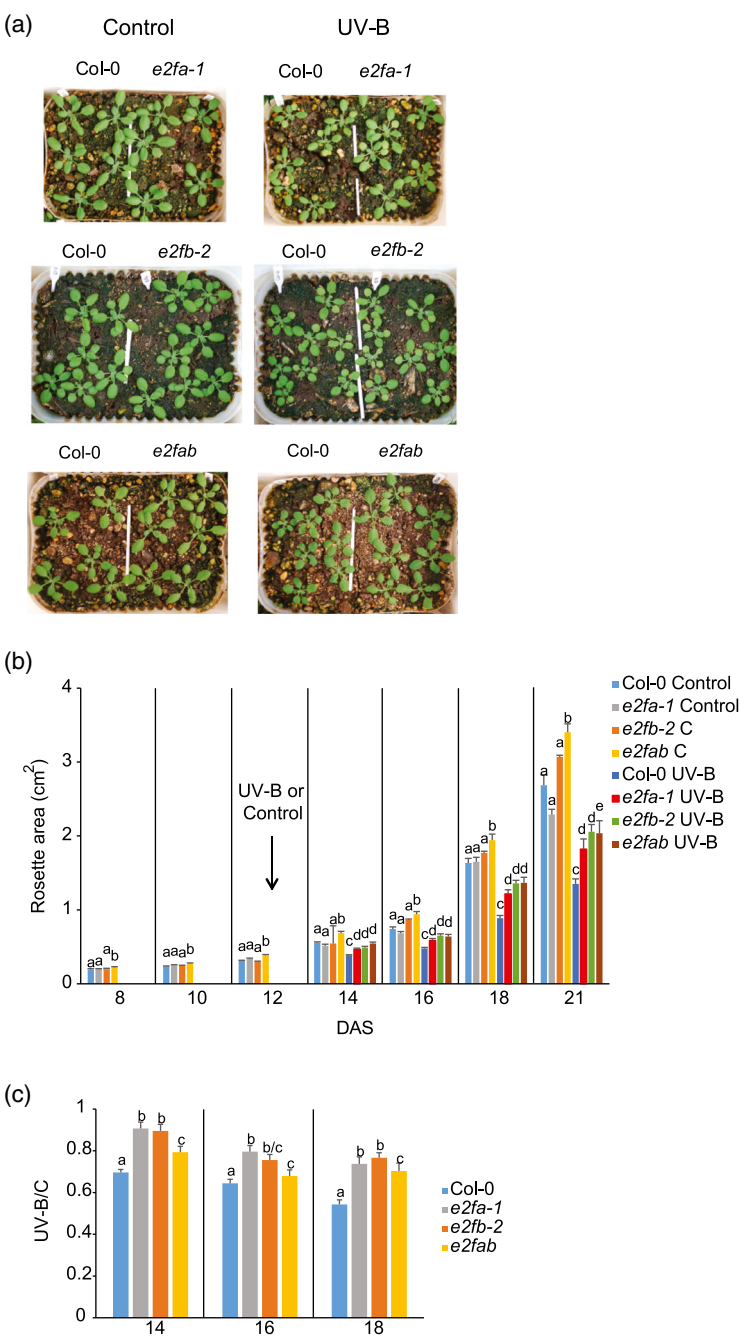

Figure 1. Ultraviolet (UV)-B differentially inhibits growth of wild-type Col-0, *e2fa*, *e2fb*, and *e2fab* Arabidopsis plants.
(a) Pictures of wild-type Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* plants that were treated with UV-B radiation for 4 h (2 W m^{-2}) or were kept under control conditions in the absence of UV-B at 12 days after sowing (DAS), 15 days after the end of the treatment.
(b) Rosette area of control and UV-B treated Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* plants measured every 2 days from day 8 until 18 DAS. Plants were UV-B treated at 12 DAS or were kept under control conditions without UV-B (indicated with an arrow). Different letters indicate statistically significant differences applying one-way (8–12 DAS) or two-way (14–18 DAS) ANOVA ($P < 0.05$).
(c) Ratio between average rosette area after UV-B exposure versus that under control conditions. Different letters indicate statistically significant differences applying one-way ANOVA ($P < 0.05$). Results represent the average of at least 20 biological replicates ± SD from one experiment. Three independent experiments were performed with similar results.

WT plants (Figure 1a–c, Figure S1a,b). In this way, *e2fb* growth is less inhibited by UV-B than WT plants.

To analyze the effect of UV-B on leaf development, the fully expanded area of leaf no. 5 was analyzed, which was emerging at the moment of the UV-B treatment. Figure 2a and Figure S2a show that leaf no. 5 from both WT and *e2fb* plants had a similar area under control conditions, and that the decrease in rosette area by UV-B was due to a decrease in final leaf area in both lines. The area of leaf no. 5 in *e2fb* mutants was bigger than that of WT plants after UV-B exposure (Figure 2a,b and Figure S2a,b), and this was because *e2fb* mutants leaves had more palisade cells (Figure 2e,f and Figure S2e,f); while the average cell area was not affected by the UV-B treatment in any of the lines (Figure 2c,d and Figure S2c,d). Thus, in UV-B-irradiated plants, *e2fb* leaf no. 5 had more cells than WT leaves, suggesting that *E2Fb* represses cell division in proliferating Arabidopsis leaves after UV-B exposure.

Because E2Fa was previously demonstrated to participate in the maintenance of genome integrity under conditions of genotoxic stress (Horvath et al., 2017), the effect of UV-B radiation was also investigated using *e2fa* mutant lines (*e2fa-1* and *e2fa-2*). Similarly, as measured for *e2fb* mutants, the rosette area of *e2fa* mutants was bigger than that from WT plants after a UV-B treatment, while the rosette area was similar in *e2fa*, *e2fb*, and WT plants under control conditions (Figure 1a,b; Figure S1a). When leaf no. 5 was analyzed, *e2fa* leaves had a similar size as WT and *e2fb* leaves, but had fewer cells with bigger areas under control conditions (Figure 2; Figure S2). After UV-B exposure, *e2fa* leaves were bigger than those from WT plants and similar to those from *e2fb* mutants (Figure 2a,b; Figure S2a,b); however, while cell size was not changed by the treatment, the decrease in cell number after UV-B was lower *in e2fa* than in WT leaf no. 5, similarly as observed in *e2fb* leaves (Figure 2c,f; Figure S2c,f). Thus, E2Fa and E2Fb both inhibit cell proliferation in the leaves after UV-B exposure, suggesting that they could have a redundant role in this process.

Changes in cell cycle progression can be assessed by analyzing transcript levels of genes where expression varies during the cell cycle (Menges et al., 2005). Therefore, we analyzed the expression of cell marker genes in WT, *e2fa*, and *e2fb* seedlings under control conditions and after UV-B exposure. mRNA levels of the cell cycle markers *CYCB1;2* (AT5G06150, encoding a cyclin required for G_2 to M progression), *KNOLLE* (AT1G08560, a G_2/M marker), and *HISTONE H4* (AT2G28740, an *S*-phase-associated gene) were quantified by reverse transcription (RT)–quantitative polymerase chain reaction (qPCR) after UV-B irradiation. Figure 3a shows that *CYCB1;2* was upregulated in seedlings from WT plants and *e2fa-1* mutants after UV-B exposure, while transcripts levels of this gene were decreased by the treatment in *e2fb-2* mutants. Interestingly, in *e2fa-1*, *CYCB1;2* levels were significantly higher than those in WT seedlings were. Besides regulating progression from G_2 to M cell cycle phases, this cyclin has also been reported to participate in the DDR (Culligan et al., 2006). Thus, increased *CYCB1;2* expression by UV-B in WT and *e2fa-1* could be related to its role during the DDR. On the contrary, *KNOLLE* levels were not changed by UV-B in WT plants, but they were increased in *e2fa-1* and decreased in *e2fb-2* seedlings (Figure 3b); while *H4* transcripts were similarly repressed after the treatment in the three lines (Figure 3c). Overall, these results suggest that the lower decrease in cell proliferation measured in the leaves of *e2fa* and *e2fb* mutants in UV-B-irradiated plants could be a consequence of altered expression and UV-B regulation of cell cycle marker genes.

In the leaves, both *E2Fa* and *E2Fb* are expressed (Arabidopsis Atlas eFP browser; Klepikova et al., 2016); thus, the role of these TFs regulating leaf growth could be redundant there. Therefore, to study further their function and possible redundancy in the responses to UV-B, experiments were also done using *e2fab* double mutants. While single *e2fa* and *e2fb* mutants had a similar rosette area as WT plants under control conditions, *e2fab* double mutants were bigger than WT and single mutants (Figure 1). After a single UV-B treatment, the double mutant showed a lower decrease in the rosette area than WT plants, and similar to that measured in *e2fa* and *e2fb* single mutants (Figure 1). Leaf no. 5 from *e2fab* double mutants was bigger than that from WT or either *e2fa* or *e2fb* single mutant plants under control conditions (Figure 2a). While leaf cells from *e2fab* mutants were bigger and less than those in WT and *e2fb* leaves were, they were similar to those in *e2fa* leaves (Figure 2c,e). Thus, this suggests that the *e2fa* mutation is epistatic over *e2fb* in the regulation of cell development in the leaves. After UV-B exposure, *e2fab* double mutants showed a similar inhibition of cell proliferation as *e2fa* or *e2fb* single mutants but lower than that measured in WT plants, without changes in cell area (Figure 2c–e). Therefore, plants deficient in the expression of both TFs still show an inhibition in cell proliferation in the leaves, suggesting that besides E2Fa and E2Fb, other proteins are required for the regulation of cell division in these tissues.

Experiments were also done using plants overexpressing *E2Fb* or *E2Fa* (*E2FbOE* and *E2FaOE*; De Veylder et al., 2002; Sozzani et al., 2006). As previously reported, *E2FbOE* plants were smaller than WT plants under control conditions (Figure S3; Sozzani et al., 2006). *E2FbOE* leaves were smaller than WT leaves because they had smaller cells, while cell number was similar between lines under control conditions (Figure S4; Oszi et al., 2020). Conversely, *E2FaOE* plants had a similar rosette area as WT plants; nevertheless, *E2FaOE* no. 5 leaves had more cells of smaller areas than WT plants (Figure S3; Figure S4). After UV-B exposure, leaf size decreased in all plants (Figure S4a,b), and although the treatment did not affect cell size in any of the three lines (Figure S4c,d), a significant decrease in cell

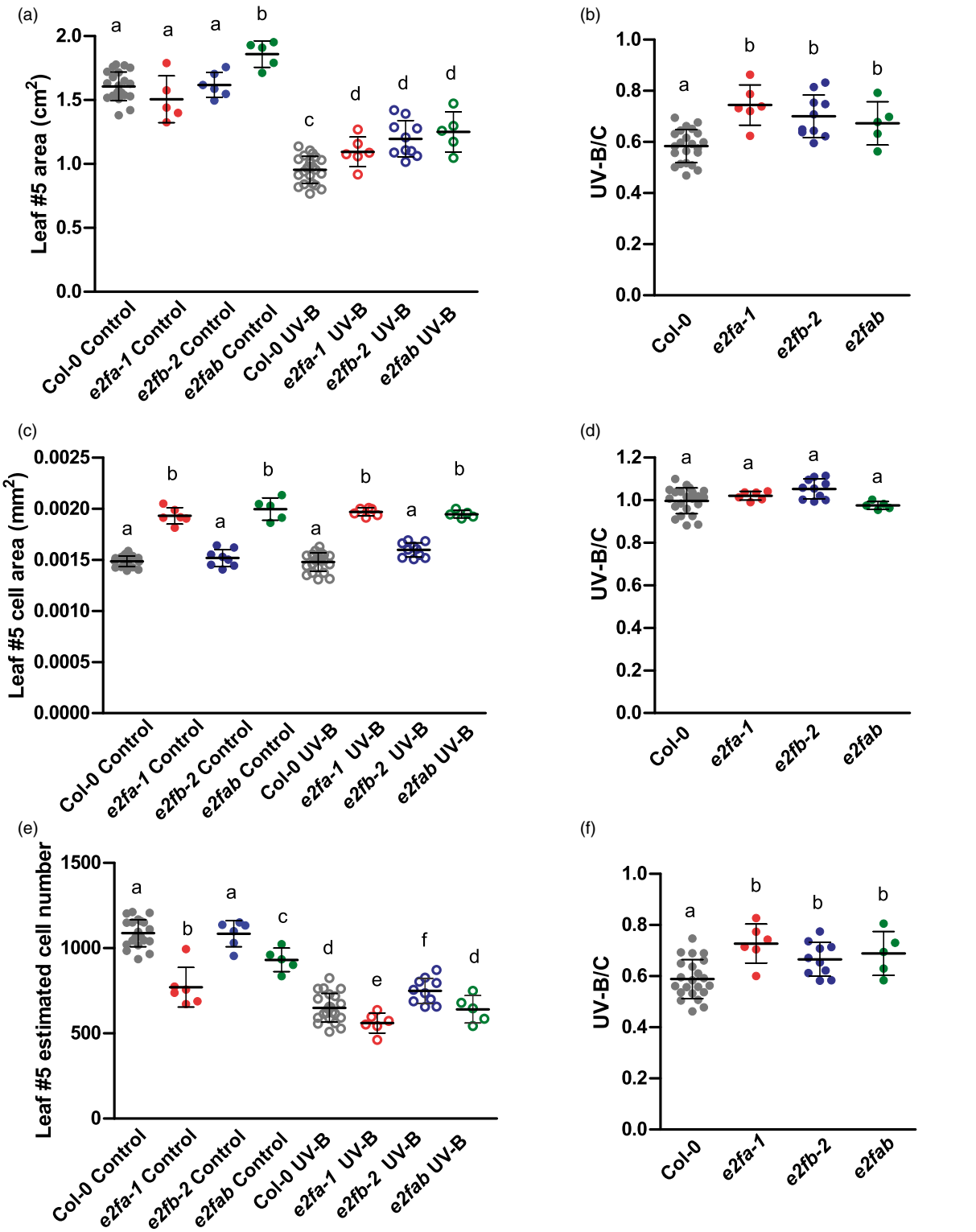

Figure 2. Ultraviolet (UV)-B differently affects cell number but not cell expansion in proliferating leaves of wild-type Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* plants.
(a) Leaf area, (c) cell area, and (e) estimated cell number of fully expanded leaf no. 5 from UV-B treated or control plants. Different letters indicate statistically significant differences applying two-way ANOVA (Tukey test, $P < 0.05$).
(b) Ratio between leaf area, (d) cell area, and (f) cell number measured of fully expanded leaf no. 5 after UV-B exposure versus under control conditions are shown. Different letters indicate statistically significant differences applying one-way ANOVA (Dunn test, $P < 0.05$). Results show the individual values and the average from at least five independent biological replicates ± SD from one experiment. Three independent experiments were performed with similar results.

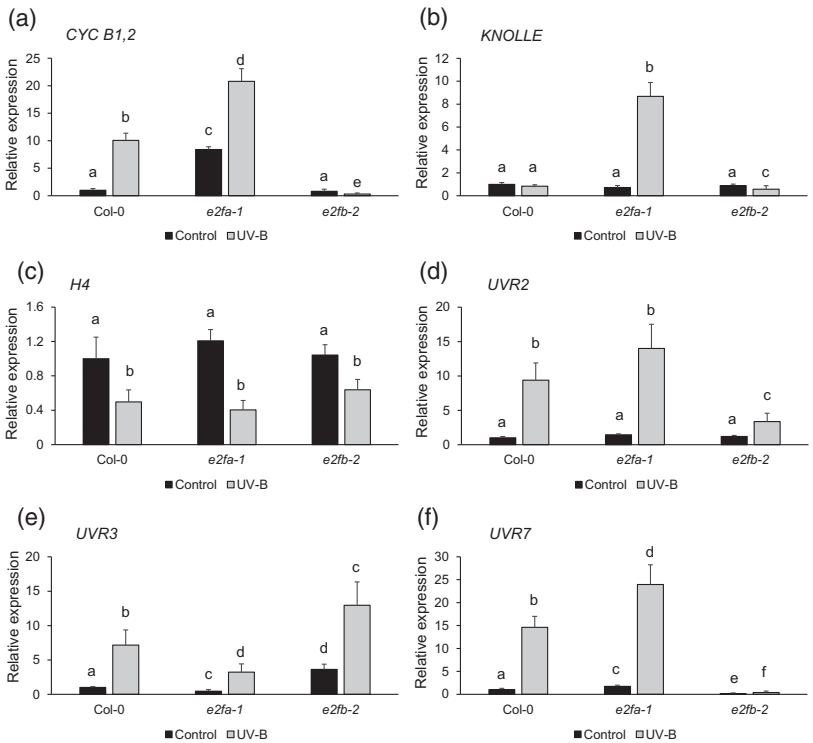

Figure 3. Expression of cell cycle markers and DNA repair genes is altered in *e2fa* and *e2fb* seedlings. Relative transcript levels of (a) *CYCB1;2*, (b) *KNOLLE*, (c) *H4*, (d) *UVR2*, (e) *UVR3*, and (f) *UVR7* genes determined by reverse transcription–quantitative polymerase chain reaction analysis in seedlings grown under control conditions in the absence of ultraviolet (UV)-B, or immediately after a 4 h UV-B treatment (2 W m^{-2}). Results represent the average of three independent biological replicates ± SEM. Different letters indicate statistically significant differences applying two-way ANOVA (Tukey test, $P < 0.05$).

number was observed, showing a similar response in all lines after UV-B exposure (Figure S4e,f). Thus, as observed in *E2Fc*-overexpressing plants (Gómez et al., 2019), overexpression of *E2Fa* or *E2Fb* did not change UV-B inhibition of Arabidopsis growth. In this way, a threshold level of these TFs seems to be enough to modulate growth after UV-B exposure. Conversely or additionally, this lack of differential UV-B response could be due to the requirement of an interaction partner expressed at similar increased levels, or the existence of a suboptimal post-translational regulatory mechanism in the *OE* plants.

E2Fa but not E2Fb regulates primary root elongation under UV-B conditions

We also analyzed weather E2Fa and/or E2Fb could regulate primary root growth in UV-B-irradiated seedlings. While WT and *e2fb* seedlings had primary roots with similar length, which were also similarly shortened after a UV-B treatment (Figure 4b,d,e; Figure S5a,b); *e2fa* seedlings had longer primary roots, and showed a lower inhibition of elongation after UV-B irradiation (Figure 4a,d,e; Figure S5a,b). In addition, roots from *e2fab* seedlings were similar to those from *e2fa* mutants both under control conditions and after a UV-B treatment (Figure 4c–e). Therefore, both E2Fb and E2Fa regulate leaf growth in plants exposed to UV-B, whereas E2fa but not E2Fb regulates inhibition of primary root elongation after UV-B exposure.

When experiments were done using seedlings overexpressing *E2Fa* or *E2Fb*, neither of these plants showed differences in the inhibition of primary root elongation compared with WT seedlings after a UV-B treatment (Figure S5c,d); despite that, *E2FbOE* seedlings had shorter primary roots than WT plants, as previously reported (Sozzani et al., 2006). Thus, similarly as in leaves, *E2Fa* or *E2Fb* overexpression does not modify the inhibition of root growth under UV-B conditions.

To investigate if the lower inhibition of root elongation in *e2fa* seedlings could be due to differences in cell proliferation in the root meristem after UV-B exposure, we analyzed the primary root meristem size in irradiated seedlings (Figure 5). *e2fa* mutants had a larger meristematic zone in the primary root than WT plants under control conditions, showing a smaller decrease in size after a UV-B treatment than that from WT roots (Figure 5a,b). The smaller decrease in the meristem size of *e2fa* roots was because of a lower inhibition of cortex cell proliferation than in WT roots (Figure 5c,d), while the increase in cortex cell length measured in the meristems of all plants caused by UV-B was similar between lines (Figure 5e,f). On the contrary, when the consequence of UV-B exposure was investigated in *e2fb* primary root meristems, they showed a similar inhibition of cell proliferation after exposure as WT roots, while those from *e2fab* mutants were similar and responded to UV-B treatment like those from *e2fa* mutants (Figure 5). Thus, in Arabidopsis, E2Fa regulates primary root elongation, both under control conditions and after UV-B exposure; while E2Fb does not participate in the inhibition of primary root elongation by UV-B.

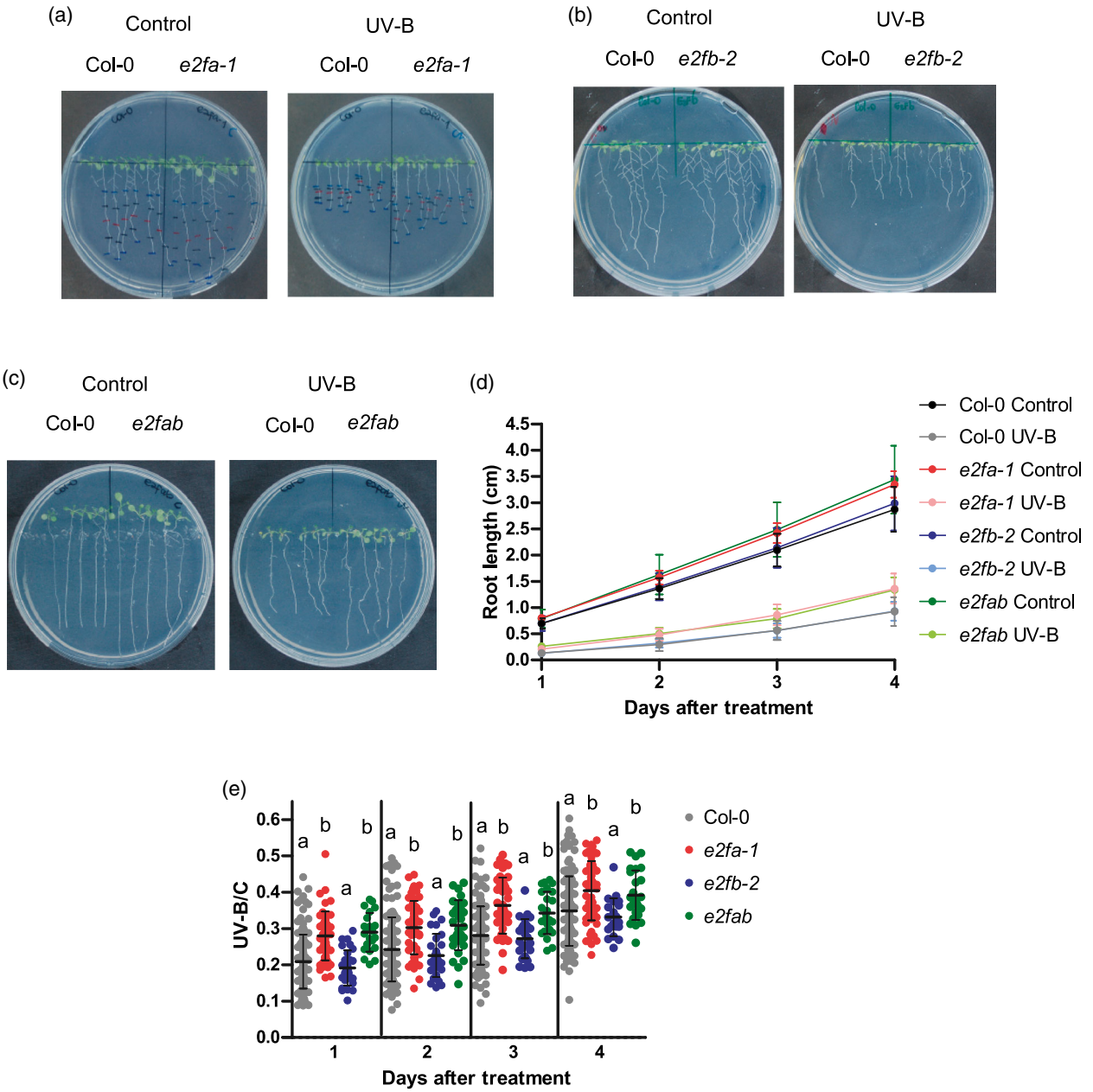

Figure 4. Primary root growth inhibition assays in Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* seedlings after ultraviolet (UV)-B exposure.
(a–c) Representative pictures of one experiment showing primary roots from control and UV-B-irradiated wild-type Col-0, (a) *e2fa-1*, (b) *e2fb-2*, and (c) *e2fab* seedlings.
(d) Graphs of average root lengths in Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* seedlings.
(e) Ratio between root lengths after UV-B exposure versus under control conditions. Results show the individual values and the average from at least 20 biological replicates ± SD from one experiment. Three independent experiments were performed with similar results. Different letters indicate statistically significant differences applying one-way ANOVA (Dunn test, $P < 0.05$).

E2Fa has a role in the activation of primary root stem cell death, but it does not participate in DNA damage repair induced by UV-B irradiation

When DNA absorbs UV-B radiation, CPDs and, to a lesser extent, pyrimidine (6-4) pyrimidone photoproducts are produced (Friedberg et al., 1995). Previous data have shown that E2Fe-deficient plants accumulate lower levels of CPDs after UV-B exposure, and this is because they express high levels of *PHR1/UVR2*, encoding a CPD photolyase (Radziejwoski et al., 2011). Interestingly, neither *e2fa*, *e2fb*, nor *e2fab* mutants showed differences in CPD accumulation after UV-B exposure compared with WT when 12-day-old

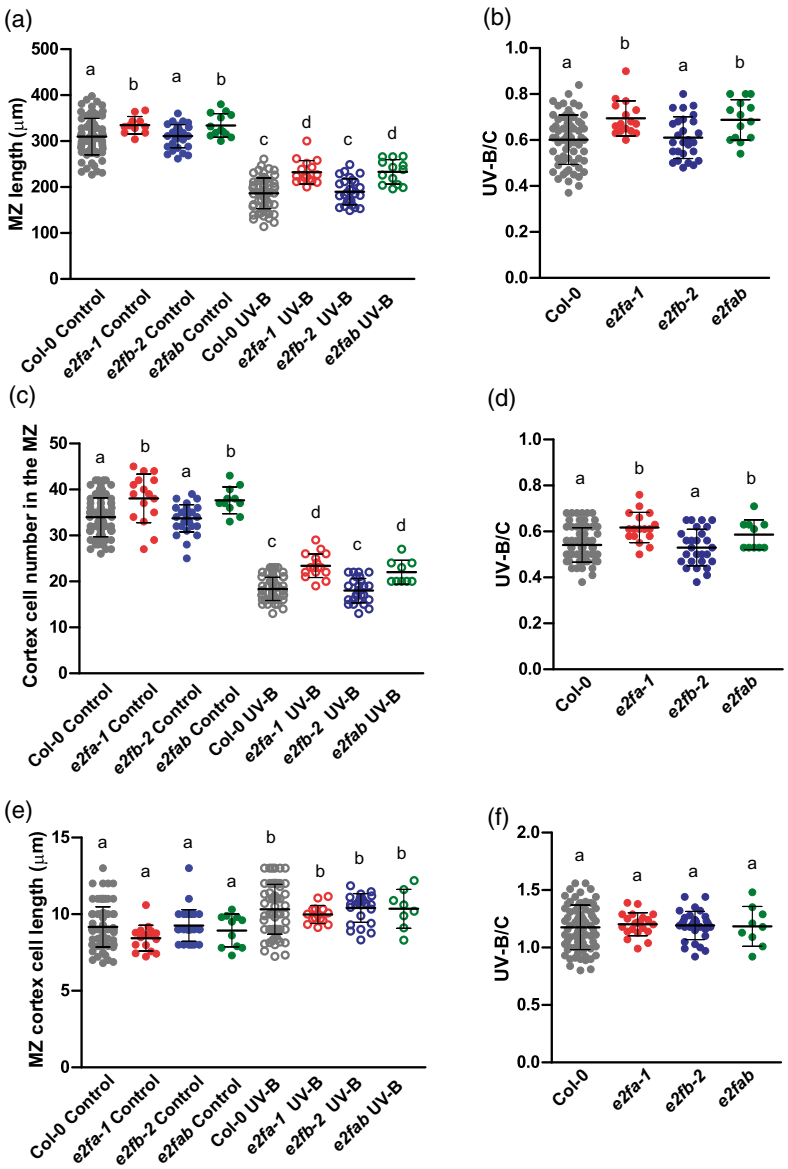

Figure 5. Ultraviolet (UV)-B differently affects cell proliferation in the root meristematic zone (MZ) of *e2fa* and *e2fab* seedlings.
(a) Root MZ length, (c) cortex cell number, and (e) cortex cell length in the root meristem from UV-B treated or control wild-type Col-0, *e2fa-1, e2fb-2*, and *e2fab* seedlings. Different letters indicate statistically significant differences applying two-way ANOVA (Tukey test, $P < 0.05$).
(b) Ratio between MZ length, (d) cortex cell number, and (f) cortex cell area values measured after UV-B exposure versus those under control conditions in primary roots are shown. Different letters indicate statistically significant differences applying one-way ANOVA (Dunn test, $P < 0.05$). Results show the individual values and the average from at least eight independent biological replicates ± SD from one experiment. Three independent experiments were performed with similar results.

plants were exposed to a single dose of UV-B for 4 h (Figure S6). Similarly, control plants that were irradiated with the same lamps also covered with a polyester plastic that absorbs UV-B showed similar and very low levels of CPDs. As shown in Figure 3d, *UVR2* was similarly expressed in WT, *e2fa-1*, and *e2fb-2* seedlings grown in the absence of UV-B, and it was upregulated after UV-B exposure in the three lines. Thus, the regulation of UV-B damage responses by E2Fa and E2Fb are independent of *UVR2* expression levels and it does not involve their participation during DNA repair, in contrast to what it was reported for E2Fe (Radziejwoski et al., 2011). On the contrary, *UVR3* (encoding a 6-4 photoproduct photolyase, AT3G15620) and *UVR7* (encoding ERCC1, a DNA excision repair protein of the nucleotide excision repair system, AT3G05210), which also encode proteins that participate in DNA damage repair after UV-B exposure, showed altered levels in both *e2fa-1* and *e2fb-2* seedlings, but in all lines they were upregulated by UV-B (Figure 3e,f). Therefore, despite the differences in expression levels of these DNA repair proteins in the mutants regarding WT seedlings, increased levels of these transcripts after UV-B exposure, together with *UVR2*, are sufficient for proper UV-B-damaged DNA repair in *E2Fa*- and *E2Fb*-deficient plants.

One of the plant responses during the DDR is the activation of PCD in meristematic tissues. After UV-B exposure, in WT plants, death of vascular stem cells takes place, which is not observed in non-treated plants (Furukawa et al., 2010). Dead cells are stained when incubated with propidium iodide (PI), while live cells exclude this compound

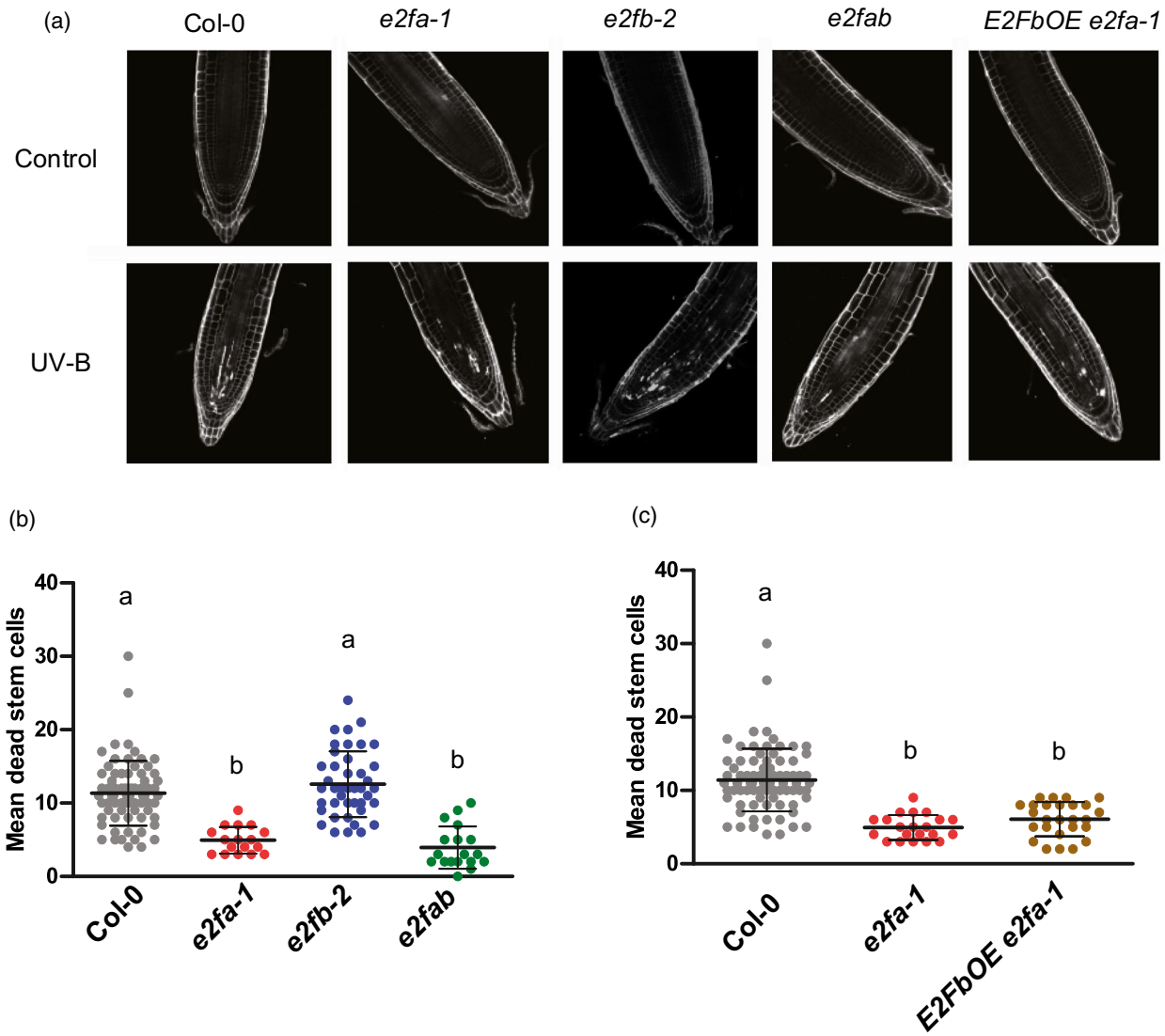

Figure 6. Ultraviolet (UV)-B affects programmed cell death in the root meristematic zone of *e2fa* and *e2fab* seedlings.
(a) Representative images of primary roots of wild-type Col-0, *e2fa-1*, *e2fb-2*, *e2fab*, and *E2FbOE e2fa-1* seedlings in which stem cells and adjacent daughter cells were propidium iodide staining to count dead stem cells per root after UV-B exposure.
(b) Number of stem cells that are dead after UV-B exposure in WT Col-0, *e2fa-1*, *e2fb-2*, and *e2fab* primary root meristems.
(c) Number of stem cells that are dead after UV-B exposure in wild-type Col-0, *e2fa-1* and *E2FbOE e2fa-1* primary root meristems. Results show the individual values and the average from at least 15 independent biological replicates ± SD from one experiment. Different letters represent statistically significant differences applying a mixed generalized linear model with a Poisson distribution ($P > 0.05$). Three independent experiments were performed with similar results.

(Furukawa et al., 2010). Thus, to investigate if E2Fa and/or E2Fb have a role in the induction of PCD in response to UV-B radiation in Arabidopsis, we quantified cell death in the primary root tips. Five-day-old seedlings vertically grown on Murashige and Skoog (MS)-agar plates were exposed to UV-B radiation for 1 h (2 W m^{-2}) and then moved to a growth chamber for 24 h in the absence of UV-B. *e2fa* and *e2fab* root tips showed significantly fewer dead cells compared with WT roots, while *e2fb* mutants showed a similar number of them as WT (Figure 6a–c). In non-irradiated plants, none of the lines showed dead cells (Figure 6a). Thus, E2Fa but not E2Fb has a role in PCD in the roots after UV-B exposure in Arabidopsis.

E2Fa and E2Fb have independent and non-complementary roles during the DDR after UV-B exposure in Arabidopsis

Our analysis showed that, while in proliferating leaves both E2Fa and E2Fb regulate the inhibition of cell proliferation, only E2Fa participates in this process in the primary root. Interestingly, *E2Fa* is highly expressed in the root meristem, becoming gradually weaker, but still detectable at the transition zone, where cells leave proliferation and

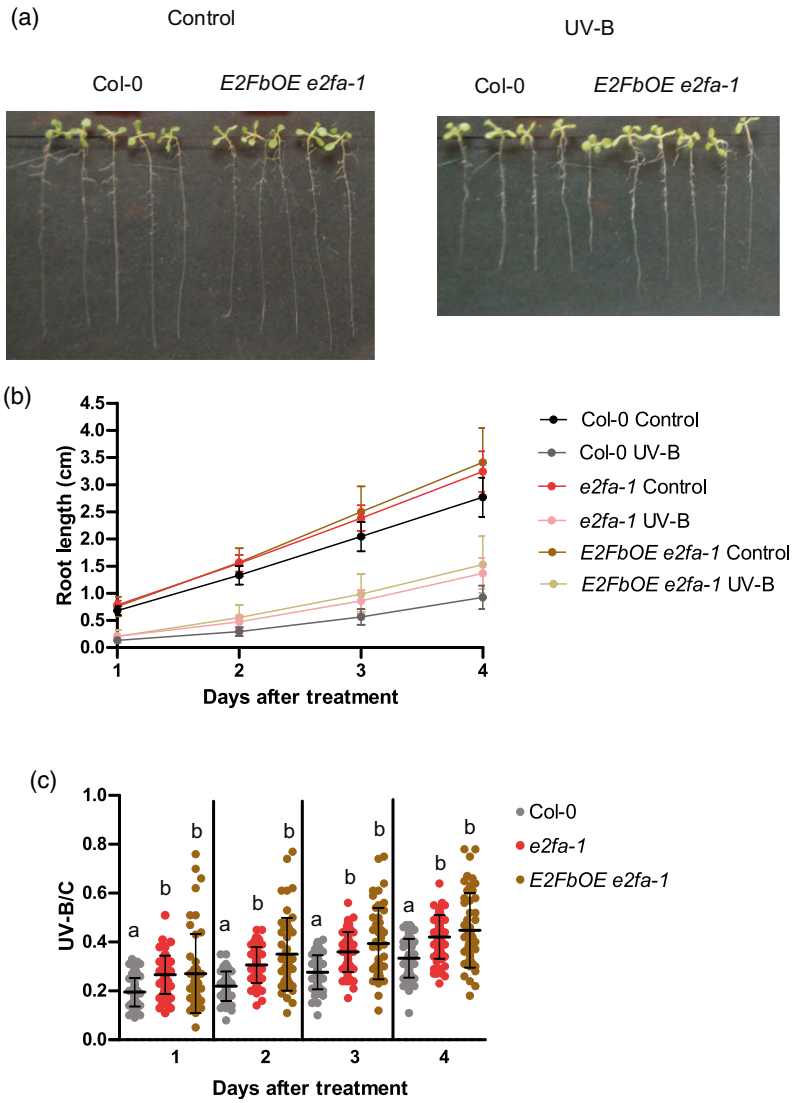

Figure 7. Primary root growth inhibition assays in Col-0, *e2fa-1*, and *E2FbOE e2fa-1 Arabidopsis* plants.
(a) Representative pictures of one experiment.
(b) Graph of average root lengths in Col-0, *e2fa-1*, and *E2FbOE e2fa-1* seedlings.
(c) Ratio between primary root length after ultraviolet (UV)-B exposure versus that under control conditions are shown. Results show the individual values and the average from at least 20 biological replicates ± SD from one experiment. Three independent experiments were performed with similar results. Different letters indicate statistically significant differences applying one-way ANOVA (Dunn test, $P < 0.05$).

start elongation in primary roots, while *E2Fb* expression is low in these zones and particularly expressed in the elongation zone (Magyar et al., 2012; Sozzani et al., 2006; Arabidopsis Atlas eFP browser; Klepikova et al., 2016). Therefore, it is possible that the lack of a differential response to a UV-B treatment in *e2fb* roots could be due to the low expression of *E2Fb* in the meristematic zone of the roots. Therefore, to analyze if the function of *E2Fa* could be complemented by ectopic expression of *E2Fb* in the roots, we generated transgenic plants that overexpressed *E2Fb* in an *e2fa* mutant background (*E2FbOE e2fa-1*). *E2FbOE e2fa-1* seedlings looked similar to WT seedlings under control conditions (Figure 7a). However, when UV-B exposed seedlings were analyzed, *E2FbOE e2fa-1* plants showed a lower inhibition of primary roots elongation than WT seedlings (Figure 7b,c). This response to UV-B was similar to that observed in *e2fa* mutants (Figure 7b,c), suggesting that increased *E2Fb* expression could not restore the function of E2Fa in irradiated roots. Moreover, the primary root meristems from *E2FbOE e2fa-1* seedlings are similar to *e2fa* meristems under control conditions and after the UV-B treatment; showing a lower inhibition of cortex cell proliferation and a lower number of dead cells after the UV-B irradiation (Figure 8; Figure 6a,d,e). Overall, these results show that, after UV-B exposure, E2Fa function in the meristematic zone of the primary roots could not be replaced by E2Fb, demonstrating that both TFs have independent and non-redundant roles in UV-B responses in Arabidopsis roots.

Activation of the DDR requires Suppressor of Gamma Response 1 (AT1G25580, SOG1; Furukawa et al., 2010), a TF that regulates the expression of genes that encode proteins in this pathway (Bourbousse et al., 2018). The activation of the DDR also requires the action of two protein kinases, Ataxia Telangiectasia Mutated (AT3G48190, ATM), and ATM and Rad3-related (AT5G40820, ATR). Thus, we

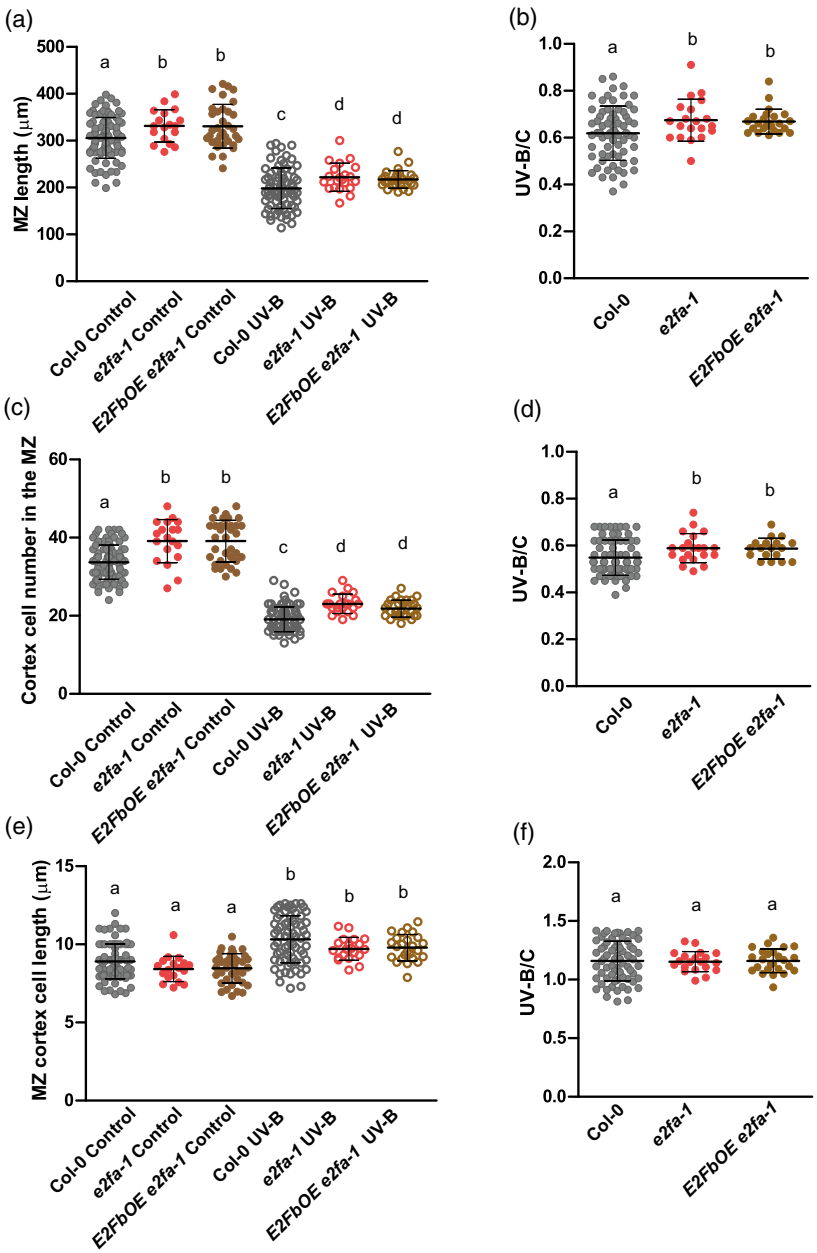

Figure 8. Ultraviolet (UV)-B affects cell proliferation in the primary root meristematic zone (MZ) of *E2FbOE e2fa-1* in a similar way as *in e2fa-1* seedlings.
(a) MZ length, (c) cortex cell number, and (e) cortex cell length in the root meristem from UV-B treated or control Col-0, *e2fa-1*, and *E2FbOE e2fa-1* seedlings. Different letters indicate statistically significant differences applying two-way ANOVA (Tukey test, $P < 0.05$).
(b) Ratio between MZ length, (d) cortex cell number, and (f) cortex cell area values measured after UV-B exposure versus those under control conditions are shown. Different letters indicate statistically significant differences applying one-way ANOVA (Dunn test, $P < 0.05$). Results show the individual values and the average from at least eight independent biological replicates ± SD. Three independent experiments were performed with similar results.

analyzed if differences in the DDR after UV-B exposure in *E2Fa*- and *E2Fb*-deficient plants could be due to changes in the expression patterns of genes encoding these proteins. We previously showed that *E2Fc*-deficient plants have altered expression of both *SOG1* and *ATR*, and *in silico* analysis of the promoter regions of these genes, showed that both of them have putative E2F consensus binding sites (Gómez et al., 2019). In our experiments, while transcript levels of these three proteins were in general similar in WT plants and *e2fb* mutants, both under control conditions and after UV-B exposure, except *SOG1* (levels were more increased by UV-B in *e2fb* mutants than in WT plants), expression of all these genes was significantly altered in *e2fa* mutants (Figure 9). Interestingly, transcript levels of *SOG1*, *ATM*, and *ATR* were significantly higher in *e2fa* mutants compared with WT or *e2fb* plants under control conditions, suggesting that E2Fa may be a negative regulator of these genes. After UV-B exposure, while the expression of *ATM* and *SOG1* was significantly repressed, ATR was highly upregulated in *e2fa* mutants (Figure 9). Therefore, UV-B regulation is also affected in *e2fa* mutants. Overall, these results suggest that altered DDR in the *e2fa* mutant could be a consequence of differences in expression patterns of the master regulators of this pathway. On the other hand, because in *e2fb* mutants, only *SOG1* levels among three tested genes were changed after UV-B

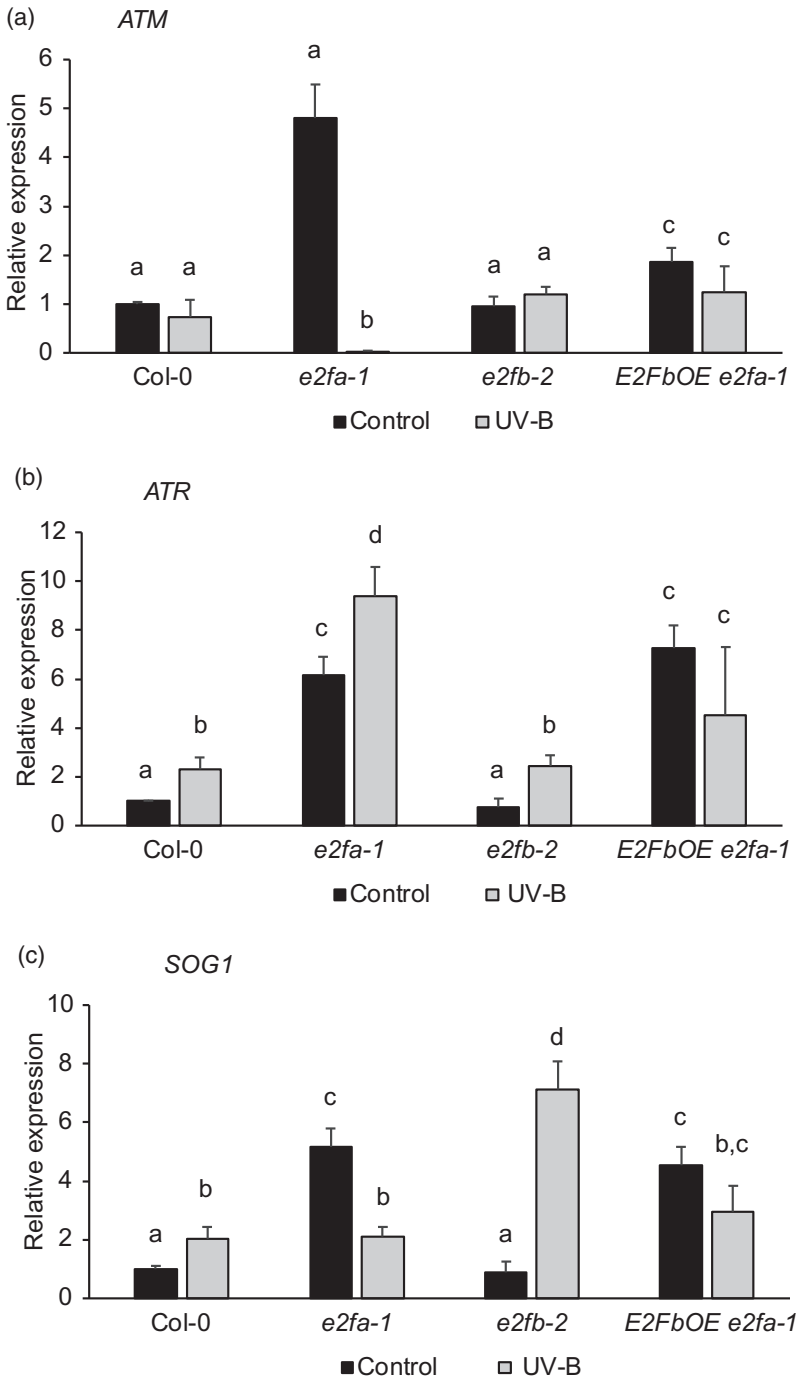

Figure 9. Ultraviolet (UV)-B effect on expression levels of DNA damage response genes in Col-0, *e2fa-1*, *e2fb-2*, and *E2FbOE e2fa-1* seedlings.
Relative expression levels of (a) *ATM*, (b) *ATR*, and (c) *SOG1* genes determined by reverse transcription–quantitative polymerase chain reaction analysis in seedlings grown under control conditions in the absence of UV-B, or immediately after a 4-h UV-B treatment at 2 W m^{-2}. Results represent the average of three biological replicates ± SEM. Different letters indicate statistically significant differences applying two-way ANOVA (Tukey test, $P < 0.05$).

exposure, altered UV-B responses in this mutant might be because of increased *SOG1* expression. Finally, and in agreement with the results shown in Figures 7 and 8, plants that overexpressed *E2Fb* in the *e2fa* mutant background could not restore WT expression levels of *SOG1*, *ATM*, or *ATR*. This result validates the different and non-redundant roles of E2Fa and E2Fb during the DDR after UV-B exposure in Arabidopsis.

DISCUSSION

We have recently shown that, in Arabidopsis and maize, UV-B radiation at intensities that produce DNA damage inhibits plant growth (Casadevall et al., 2013; Fina et al., 2017; Gómez et al., 2019). In particular, leaf and root growth is impaired because UV-B inhibits cell proliferation in developing organs. Interestingly, the inhibition of cell

proliferation by UV-B is in part regulated by the miR396/GROWTH REGULATING FACTORs (GRFs) pathway (Casadevall et al., 2013; Fina et al., 2017). In Arabidopsis and maize, the microRNA miR396 levels are increased after a UV-B treatment in proliferating tissues, and consequently, there is a decrease in *GRF1*, *GRF2*, and *GRF3* levels, inhibiting cell proliferation (Casadevall et al., 2013; Fina et al., 2017).

In addition, plant growth under UV-B conditions is also controlled by some members of the E2F TF family (Gómez et al., 2019; Radziejwoski et al., 2011). In particular, E2Fc and E2Fe regulate cell proliferation in UV-B-irradiated Arabidopsis plants (Gómez et al., 2019; Radziejwoski et al., 2011). *E2Fe* is a target of regulation of both E2Fb and E2Fc, which compete for a single *cis*-acting binding site in response to light conditions (Berckmans et al., 2011). Despite this, E2Fc regulates plant growth under UV-B conditions differently as E2Fe. While growth of *E2Fc*- and *E2Fe*-deficient plants is less inhibited by UV-B than WT plants, *e2fe* mutant leaves are bigger than WT leaves after UV-B exposure mainly because they have bigger cells (Radziejwoski et al., 2011). Contrarily, leaves from *E2FcRNAi* plants have more cells than WT leaves after UV-B exposure (Gómez et al., 2019). Moreover, *e2fe* leaf cells have altered ploidy levels and, because this TF negatively controls the expression of the photolyase UVR2, *e2fe* plants accumulate lower CPDs levels than WT plants after UV-B exposure, whereas *E2Fc*-deficient leaves do not show differences in cell ploidy or DNA damage compared with WT leaves (Gómez et al., 2019; Radziejwoski et al., 2011).

Because E2Fb is also a transcriptional regulator of *E2Fe*, we analyzed its role in UV-B responses. Similarly, as previously described for *e2fe* mutants, proliferating leaves from *e2fb* plants had a similar area as WT leaves under control conditions, but a smaller decrease in leaf size and cell number after UV-B exposure (Radziejwoski et al., 2011; Figure 2). However, *e2fe* leaves also showed an increase in cell size, probably due to changes in ploidy levels after UV-B exposure (Radziejwoski et al., 2011), while cell area and ploidy levels in the leaves of *e2fb* mutants were not changed by the UV-B treatment (Figure 2; Figure S7). Thus, the regulation of cell proliferation mediated by E2Fe after exposure could be due to its regulation by E2Fb, but not changes in the endoreduplication index. Moreover, while *e2fb* mutants accumulate similar levels of CPDs after UV-B as WT plants, *e2fe* mutants show less DNA damage. Interestingly, *e2fb* seedlings show an upregulation of DNA repair transcripts after UV-B exposure, including *UVR2* (Figure 3). Thus, while some UV-B responses in Arabidopsis that require E2Fe are also similarly regulated by E2Fb and could be through the regulation of this TF, others are independent of E2Fb.

When we analyzed developmental responses in *e2fa* mutants, the results showed that under control conditions, leaves had fewer but larger cells than WT plants; however, after UV-B exposure, they showed a smaller decrease in cell proliferation without changes in the cell area. Therefore, we showed that both E2Fa and E2Fb act as negative regulators of cell proliferation in Arabidopsis leaves exposed to UV-B. Our results show that in *e2fa* and *e2fb*, the expression of cell cycle markers was altered after UV-B exposure (Figure 3). *CYCB1;2*, which shows a maximal expression at the G_2/M transition, was upregulated in seedlings from WT plants and *e2fa-1* mutants but not in *e2fb-2* mutants after UV-B exposure; and in *e2fa-1*, its levels were significantly higher than those in WT seedlings. On the other hand, *KNOLLE*, which peaks during mitosis, was not changed by UV-B in WT plants, but it was increased in *e2fa-1* and decreased in *e2fb-2* seedlings, while *H4*, which is associated to the *S*-phase, was similarly repressed by UV-B in the three lines (Figure 3). Taken together, these findings indicate that the regulation of cell proliferation in UV-B-irradiated plants by E2Fa and E2Fb may be altered because expression and UV-B regulation of cell cycle marker genes is affected.

Interestingly, leaves from *e2fab* double mutants were bigger under control conditions with fewer cells and larger areas than WT leaves. This phenotype is similar to that from *e2fa* single mutant leaves, while those from *e2fb* mutants were similar to WT leaves, suggesting that the *e2fa* mutation is epistatic over *e2fb* in the regulation of leaf cell development. This phenotype was similar to that reported during embryo development (Leviczky et al., 2019). After UV-B exposure, *e2fab* double mutants also showed a similar inhibition of cell proliferation as *e2fa* and *e2fb* single mutants. Even though the single and double mutants show a lower inhibition of cell proliferation after UV-B exposure, there is still a significant decrease in cell number in *e2fab*-irradiated plants, suggesting that other pathways than those regulated by both E2F TFs are required to regulate cell proliferation under UV-B conditions.

On the other hand, our results show that despite both E2Fa and E2Fb regulate cell proliferation under UV-B conditions in the leaves, only E2Fa regulates primary root elongation and cell proliferation in the meristems of irradiated plants. While E2Fa has an important role in the root, *E2Fb* is not highly expressed in the proliferative zone (Horvath et al., 2017; Arabidopsis Atlas eFP browser; Klepikova et al., 2016; Sozzani et al., 2006). Interestingly, in response to UV-B, a deficiency in *E2Fa* expression could not be reverted by an overexpression of *E2Fb* in the roots (Figure 7 and 8), suggesting that the function of E2Fa in this organ in response to UV-B cannot be replaced by E2Fb, and differences cannot be only explained by expression patterns of both genes in these tissues. E2Fa differs from E2Fb in various ways; while both E2Fa and E2Fb activate *S*-phase genes; E2Fa also regulates the expression of

genes required for differentiation of post-mitotic cells (Heyman et al., 2011; Magyar et al., 2012; Naouar et al., 2009). E2Fa is mostly abundant in *S*-phase cells, while E2Fb is expressed throughout the cell cycle phases; and it can drive both the G_1-to-S and the G_2-to-M transitions, stimulating cell divisions (Magyar et al., 2000, 2005; Mariconti et al., 2002). When *E2Fa* is overexpressed, it can promote cell proliferation in meristematic cells; but in cells that have lost cell division competence, it supports endoreduplication (Magyar et al., 2012). Interestingly, E2Fa function during endoreduplication requires its association with RBR to repress genes that regulate the entry into endocycle and cell differentiation. In *E2Fb* overexpressing plants, the cell doubling time is shortened and leaves show cells with reduced size compared to WT leaves (Figure S4; Magyar et al., 2005). In our experiments, neither E2Fa nor E2Fb overexpression induced differences in the plant responses to UV-B to those in WT plants, suggesting that basal expression levels of these two TFs are sufficient to control these responses. Interestingly, a similar lack of differential response to UV-B was previously observed in experiments using E2Fc overexpressing plants (Gómez et al., 2019).

The independent and different activation of pathways by the three typical E2F TFs has been reported several times. For example, the activity of E2Fb bound to RBR is reduced in response to sucrose availability or overexpression of the cyclin CYCD3;1, while the association of E2Fa with RBR is instead enhanced when cell proliferation is induced by either of these conditions (Magyar et al., 2012; De Veylder et al., 2002). E2Fa with RBR maintains the proliferation competence by repressing genes controlling the switch from mitosis to endocycle and cell elongation (Magyar et al., 2012), while E2Fb with RBR regulates the cell cycle in a more canonical way, repressing the activation of cell cycle genes through the inhibition of E2Fb. In Arabidopsis, E2Fc, RBR, and MYB3R3 (a repressor type MYB3R or Rep-MYB3R) are part of the DREAM complex (dimerization partner [DP], RB-like, E2F and MuvB), which has a repressive function that establishes quiescence (Kobayashi et al., 2015). E2Fb, on the other hand, associates with the mitosis-specific activator MYB3R4 in a different DREAM complex (Harashima and Sugimoto, 2016; Kobayashi et al., 2015). This provides additional support for the mitotic role of E2Fb. While both E2Fb and E2Fc are components of DREAM complexes, E2Fa is not (Horvath et al., 2017; Kobayashi et al., 2015; Sadasivam and DeCaprio, 2013). On the other hand, when *E2Fb* was overexpressed together with *DPA* in cultured tobacco cells, continuous proliferation was measured (Magyar et al., 2005). Interestingly, *E2Fb* overexpression without DP, both in Arabidopsis and tomato, still showed an upregulation of cell cycle genes, suggesting that E2Fb levels are limiting (Abraham and del Pozo, 2012; Sozzani et al., 2006). On the contrary, when *E2FC* was overexpressed, there was a suppression of meristematic cell divisions and a decrease in the expression of mitotic *CYCB1;1*, while when silenced an upregulation of *S*-phase-associated *HISTONE 4*, *CELL DIVISION CYCLE 6*, and *CYCB1;1* genes were observed (del Pozo et al., 2006). Thus, RBR probably represses cell proliferation through E2Fb (Magyar et al., 2012), while E2Fc acts as a transcriptional repressor and is required for the correct end of cell division and occurrence of endoreduplication (de Jager et al., 2009; del Pozo et al., 2006). In young leaves, both RBR and E2Fb are abundant and form a repressor complex (Oszi et al., 2020). When levels of E2Fb are increased, either alone or with DPa, there is also an increase in the amount of this repressor complex, and this produces a reduction in leaf cell number. On the other hand, in *e2fb* mutants, there is an increase in cell proliferation, suggesting that RBR repression specifically acts through its interaction with E2Fb. Thus, E2Fb, in association with RBR, is a key regulator of cell proliferation during organ development, establishing final leaf cell number. Overall, our results and data from other groups reinforce the different and non-redundant roles of these TFs. Our data showed that all three typical E2F TFs in Arabidopsis play roles regulating cell proliferation in Arabidopsis plants exposed to UV-B, but their function is different in leaves and roots.

E2Fa was previously shown to play roles maintaining genome integrity and viability in meristematic cells (Horvath et al., 2017; Biedermann et al., 2017). After DNA damage with different genotoxic agents, such as mitomycin C, zeocin, or hydroxyurea; RBR and E2Fa were found to be recruited to foci with damaged DNA. These lesions were also associated with the heterochromatic mark γH2AX and to the conserved repair protein, AtBRCA1. E2Fa, together with RBR, were found to regulate the DDR and the cell death pathway transcriptionally (Horvath et al., 2017). Altered DDRs after UV-B exposure in *e2fa* mutant roots could be a consequence of endogenous DNA damage that could be activated by exposure to UV-B. A similar activation of PCD in the meristems of the primary roots has been shown after treatment with other genotoxic agents such as ionizing radiation (Furukawa et al., 2010), zeocin and bleomycin (Fulcher and Sablowski, 2009), which produce double strand breaks in the DNA. However, UV-B doses used in our experiments only induce the formation of CPD and pyrimidine (6–4) pyrimidone photoproducts (Friedberg et al., 1995; Britt, 1996); and they do not produce double strand breaks or oxidative damage in the DNA as other genotoxic agents do (Britt, 1996; Fina et al., 2017). Thus, the E2Fa role in root growth inhibition and PCD after UV-B exposure may not relate to its role during DSB repair. *rbr1* mutants also showed increased levels of DNA lesions, indicating a direct role of RBR1 in the DDR. RBR1 also physically interacts with the DNA repair protein, AtBRCA1; thus, overall, both proteins could have a role maintaining genome integrity. Moreover, RBR1 regulates cell death after DNA damage with aluminum (Biedermann et al.,

2017). RBR1 acts as a repressor of DDR genes such as *RADIATION SENSITIVE 51* (*RAD51*), and it is required for RAD51 localization to DNA lesions (Biedermann et al., 2017). In our experiments, *e2fa* and *e2fab* but not *e2fb* mutants showed decreased number of dead cells in the primary root meristem after a UV-B treatment compared with WT seedlings. In this way, besides regulating cell proliferation, E2Fa participates in other aspects of the DDR such as in the activation of PCD. Interestingly, none of the mutants analyzed in this work showed differences in the accumulation of DNA damage after UV-B exposure, in contrast to what was previously reported in experiments using *e2fe* mutants (Radziejwoski et al., 2011).

When we analyzed how transcripts encoding proteins participating in the activation of the DDR were accumulated in WT and plants with altered expression of *E2Fa* and/or *E2Fb*, expression patterns of *ATM*, *ATR*, and *SOG1* were changed compared with those of WT levels. In *e2fb* mutants, only *SOG1* levels were increased more by UV-B than in WT plants; while expression of all three genes was significantly altered in *e2fa* mutants. Interestingly, transcript levels of *SOG1*, *ATM*, and *ATR* were significantly higher in *e2fa-1* mutants compared with WT under control conditions; this increased expression parallels the higher expression of *AtBRCA1*, encoding a conserved DNA repair protein, previously reported in this mutant (Horvath et al., 2017). Thus, E2Fa probably acts as a negative regulator of DDR genes. After UV-B exposure, the expression of *ATM* and *SOG1* was significantly repressed in *e2fa-1* mutants, while ATR was highly upregulated. Interestingly, the upregulation of At*BRCA1* by the DNA cross-linker mitomycin was also lower in *e2fa-1* compared with WT plants, but it was not changed in *e2fb* mutants (Horvath et al., 2017). Thus, UV-B regulation response is also affected in *e2fa* mutants, suggesting that differences in the DDR after UV-B exposure in *e2fa* mutants could be a consequence of altered expression patterns of the master regulators of this pathway. On the contrary, in *e2fb* mutants, changes in UV-B responses measured could be due to high *SOG1* expression, and/or decreased expression of the cell cycle markers *CYCB1,2* and *KNOLLE* after the treatment. The lack of reversion of WT expression levels of *SOG1*, *ATM*, or *ATR* in plants overexpressing *E2Fb* in the *e2fa-1* mutant background again validates the different and non-redundant roles of E2Fa and E2Fb during the DDR after UV-B exposure in Arabidopsis. Interestingly, we previously demonstrated that *E2Fc*-deficient plants had higher *SOG1* and *ATR* expression under control conditions compared with WT plants, without showing upregulation after UV-B exposure (Gómez et al., 2019). In this way, despite that at least four of the six characterized E2F TFs in Arabidopsis participate in responses to UV-B irradiation, each of them seems to have different roles during the DDR. It is interesting to note that *in silico* analysis showed that both *SOG1* and *ATR* have putative E2F consensus binding sites in their promoters (Gómez et al., 2019), suggesting that different E2Fs could differentially regulate these genes under genotoxic conditions, competing for the same binding sites.

In conclusion, in this study, we demonstrate that at UV-B intensities that induce DNA damage, inhibition of cell proliferation is regulated by both E2Fa and E2Fb in the leaves, while only E2Fa regulates the DDR in the roots. The role of E2Fa in the roots could not be replaced by E2Fb, and both TFs have different and non-redundant roles in developmental and DDRs in Arabidopsis plants exposed to UV-B radiation.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions, and irradiation protocols

Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments. The T-DNA insertion mutants *e2fb-1* (SALK_103138) and *e2fb-2* (SALK_131064) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA), while *e2fa-1* (MPIZ-244), *e2fa-2* (GABI_348E09) were provided by Dr. Lieven De Veylder (VIB-UGent Center for Plant Systems Biology, Belgium). The *E2FaOE* seeds stock was provided by Dr. Lieven De Veylder and *E2FbOE* seed stock was provided by Dr. Crisanto Gutierrez (Centro de Biología Molecular Severo Ochoa, Spain). The *e2fa-2 e2fb-1* double mutant seeds (*e2fab*) were described in Heyman et al. (2011) and they were provided by Dr. Lieven De Veylder. *E2FbOE e2fa-1* plants were obtained by crossing single homozygous *e2fa-1* mutants and overexpressing *E2FbOE* plants to obtain F_2 progenies that were genotyped by PCR amplification of genomic DNA with specific primers that amplify the 35S promoter from the *E2FbOE* transgene, and the T-DNA insertion in the *E2Fa* gene (Table S1).

For most experiments, Arabidopsis plants were grown on soil at 22°C under a 16 h/8 h light/dark photoperiod (100 $\mu Em^{-2}sec^{-1}$). Plants were exposed for 4 h to UV-B using fixtures mounted 30 cm above the plants (2 W m^{-2} UV-B and 0.6 W m^{-2} UV-A; Bio-Rad ChemiDoc^{TM} XRS UV-B lamps, catalog 1708097). The lamps have a peak at 302 nm and an emission spectrum from 290–310 nm, and they were covered using cellulose acetate filters (100 mm extra-clear cellulose acetate plastic; Tap Plastics, Mountain View, CA, USA). The cellulose acetate filter absorbs wavelengths lower than 290 nm; this control was done in case some lower wavelength radiation was produced with lamps aging. As a control without UV-B, plants were exposed for the same time under the lamps also covered with a polyester plastic that absorbs UV-B at wavelengths lower than 320 nm (PE, 100 mm clear polyester plastic; Tap Plastics). UV and white light radiation were recorded using a UV-B/UV-A radiometer (UV203 AB radiometer; Macam Photometrics, Glasgow, UK), and a laboratory Quantum Scalar Irradiance Meter (Biospherical Instruments QSL-100, San Diego, CA, USA), respectively. Samples were collected immediately after the light treatments.

For primary root analysis, Arabidopsis seedlings were grown in Petri dishes. Sterilized seeds were grown on MS growth medium supplemented with 0.7% agar and were kept in a vertical position in the growth chamber. After 5 days of growth, seedlings were irradiated with the same UV-B lamps for 1 h (2 W m^{-2} UV-B) and then kept without UV-B in the growth chamber.

Quantitative RT-PCR

Total RNA purification and qRT-PCR was done as described in Casadevall et al. (2013). Primers for each transcript under study were designed using the PRIMER3 software (Rozen and Skaletsky, 2000) to amplify unique 150–250 bp product (Table S1). Transcript levels were normalized to those of the *A. thaliana* calcium-dependent protein kinase3 (*CPK3*, Table S1).

DNA damage analysis

CPDs were quantified by dot-blot analysis using monoclonal antibodies (TDM-2; Cosmo Bio Co., Ltd., Japan). Twelve-day-old plants were treated with UV-B during 4 h, and samples (0.1 g) were collected immediately after the treatment, immersed in liquid nitrogen and stored at −80°C. Two micrograms of the extracted DNA using a modified cetyltrimethylammonium bromide method was then denatured using 0.3 M NaOH for 10 min. Samples were analyzed by dot blot in sextuplicate using a nylon membrane (Perkin Elmer life Sciences, Inc., Waltham, MA, USA). The membrane was then incubated at 80°C for 2 h and then blocked with a buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl (TBS) and 5% (p/v) dried milk for 1 h at room temperature. After this, the membrane was washed with TBS and incubated with anti-CPDs antibodies (1:2000 in TBS) overnight at 4°C with agitation. Unbound antibodies were washed away and secondary antibodies conjugated to alkaline phosphatase (1:3000; Bio-Rad, Hercules, CA, USA) were added. The blot was washed three times and it was developed by the addition of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. Quantification was done by densitometry using ImageQuant software version 5.2. Total DNA was quantified fluorometrically using the Qubit dsDNA assay kit (Invitrogen, Waltham, MA, USA), and checked in a 1% (w/v) agarose gels after quantification.

Root length measurements

Seedlings were grown in MS-agar plates for 5 days, and then they were UV-B-irradiated for 1 h and kept in the absence of UV-B for 4 days. Plates were photographed before and 1, 2, 3, and 4 days after the treatment, and the images were examined using the ImageJ software version 1.52 p.

Root meristem analysis and PCD after UV-B exposure

Seedlings were grown in MS-agar plates, and 5 days after stratification they were UV-B-irradiated. Then, seedlings were kept for 24 h under control conditions in the growth chamber, and PCD was analyzed by staining the root tips with a modified pseudo-Schiff PI staining protocol (Furukawa et al., 2010). Primary roots were observed and photographed by confocal laser scanning microscopy (Nikon C1, Tokyo, Japan) under water with a 40× objective. The excitation wavelength for PI-stained samples was 488 nm and emission was collected at 520–720 nm. Dead cells in the meristematic zone, which show intense PI staining, were analyzed using the ImageJ software version 1.52 p.

Rosette area quantification

Twenty seeds were sown per tray without superposition during plant growth. Twelve days after sowing, a group of plants were exposed to UV-B radiation during 4 h at 2 W m^{-2}, while a second group was kept as control and was not irradiated. After the treatments, the plants were kept in a growth chamber in the absence of UV-B. Pictures were taken every 2 days, and total leaf or rosette area of each plant was quantified using the ImageJ software version 1.52 p.

Microscopic observations

Leaves were fixed using a solution with 50% (v/v) ethanol; 5% (v/v) acetic acid and 3.7% (v/v) formaldehyde; and then they were cleared with a solution containing 200 g chloral hydrate, 20 g glycerol, and 50 ml dH_2O (Horiguchi et al., 2005). Leaf images were acquired with a differential interference contrast microscopy; and areas were quantified using the ImageJ software version 1.52 p. Palisade leaf cells were observed by differential interference contrast microscopy, palisade cell area was quantified, and to calculate the total number of palisade cells in the subepidermal layer, the leaf blade area was divided by cell area. Eighty palisade cells were analyzed per leaf to determine the cell area. Experiments were done in duplicate with at least 10 leaves with similar results.

Flow cytometry analysis of leaf no. 5

Ten leaves were cut with a razor blade in 1 ml of a buffer containing 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 3-[*N*-morpholino] propane-sulfonic acid, pH 7.0, and 1% Triton X-100 (Galbraith et al.,1991). The supernatant was filtered over a 30 μm mesh and 1 μl of 4′,6-diamidino-2-phenylindole from a stock of 1 mg ml^{-1} was added with 50 mg ml^{-1} RNase (Thermo-Fisher, Waltham, MA, USA). The extract was read through the Cell Sorter BD FACSAria II flow cytometer. The endoreduplication index was calculated with the formula: Endoreduplication index = [(0 × %2C)+(1 × %4C) + (2 × %8C) + (3 × %16C) + (4 × %32C)]/100 (Barrow and Meister, 2003). This experiment was done in triplicate, using 10 plants for each treatment/genotype. In every experiment, at least 5000 nuclei were analyzed.

Statistical analysis

Comparisons between different genotypes grown under the same growth condition, or UV-B versus control ratios from different lines (comparisons between one independent variable) were done using one-way ANOVA (Dunn Test). Comparisons between different genotypes and treatments were analyzed using two-way ANOVA (Tukey test), using non-transformed data. These statistical analyses were performed using Sigma Plot 7.0. Dead meristematic cells were analyzed using the mixed generalized linear model with a Poisson distribution ($P > 0.05$), these analyses were performed using Infostat.

ACCESSION NUMBER

E2Fa (AT2G36010), *E2Fb* (AT5G22220).

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AUTHORS CONTRIBUTIONS

MSG, MLS, and PC conceived and designed the study. MSG and MLS performed the experiments. MSG, MLS, and PC analyzed data. PC wrote the paper.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. UV-B differentially inhibits growth of WT Col-0, *e2fa*, and *e2fb* Arabidopsis plants.

Figure S2. UV-B differently affects cell number but not cell expansion in proliferating leaves of WT Col-0, *e2fa-2*, and *e2fb-1 A. thaliana* plants.

Figure S3. UV-B similarly affects rosette area of Col-0, *E2Fa*, and *E2Fb* overexpressing *A. thaliana* plants.

Figure S4. UV-B similarly affects cell number in proliferating leaves of Col-0, *E2FaOE*, and *E2FbOE A. thaliana* plants.

Figure S5. Primary root inhibition assays in Col-0, *e2fa-2*, and *e2fb-1*, *E2FaOE*, and *E2FbOE* seedlings after UV-B exposure.

Figure S6. Relative CPD levels in the DNA of WT Col-0, *e2fa, e2fb*, and *e2fab* plants under control conditions and immediately after a 4-h UV-B treatment.

Figure S7. Ploidy levels of cells in leaf no. 5 from Col-0 and *e2fb* plants after UV-B treatment or under control conditions.

Table S1. Primers used for RT-qPCR and PCR assays.

REFERENCES

Abraham, Z. & del Pozo, J.C. (2012) Ectopic expression of E2FB, a cell cycle transcription factor, accelerates flowering and increases fruit yield in tomato. *Journal of Plant Growth Regulation*, **31**, 11–24.

Barrow, M. & Meister, A. (2003) Endopolyploidy in seed plants is differently correlated to systematic, organ, life strategy and genome size. *Plant Cell and Environment*, **26**, 571–584.

Berckmans, B., Lammens, T., Van Den Daele, H., Magyar, Z., Bögre, L. & De Veylder, L. (2011) Light-dependent regulation of *DEL1* is determined by the antagonistic action of E2Fb and E2Fc. *Plant Physiology*, **157**, 1440–1451.

Biedermann, S., Harashima, H., Chen, P., Heese, M., Bouyer, D., Sofroni, K. et al. (2017) The retinoblastoma homolog RBR1 mediates localization of the repair protein RAD51 to DNA lesions in Arabidopsis. *The EMBO Journal*, **36**, 1279–1297.

Boniotti, M.B. & Gutierrez, C. (2001) A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in Arabidopsis, a CDKA/cyclin D complex. *The Plant Journal*, **28**, 341–350.

Bourbousse, C., Vegesna, N. & Law, J.A. (2018) SOG1 activator and MYB3R repressors regulate a complex DNA damage network in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **115**, E12453–E12462.

Britt, A.B. (1996) DNA damage and repair in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **47**, 75–100. https://doi.org/10.1146/annurev.arplant.47.1.75

Casadevall, R., Rodriguez, R.E., Debernardi, J.M., Palatnik, J.F. & Casati, P. (2013) Repression of growth regulating factors by the microRNA396 inhibits cell proliferation by UV-B radiation in Arabidopsis leaves. *The Plant Cell*, **25**, 3570–3583.

Culligan, K.M., Robertson, C.E., Foreman, J., Doerner, P. & Britt, A.B. (2006) ATR and ATM play both distinct and additive roles in response to ionizing radiation. *The Plant Journal*, **48**, 947–961.

de Jager, S.M., Scofield, S., Huntley, R.P., Robinson, A.S., den Boer, B.G.W. & Murray, J.A.H. (2009) Dissecting regulatory pathways of G1/S control in *Arabidopsis*: common and distinct targets of CYCD3;1, E2Fa and E2Fc. *Plant Molecular Biology*, **71**, 345–365.

De Veylder, L., Beeckman, T., Beemster, G.T., de Almeida Engler, J., Ormenese, S., Maes, S. et al. (2002) Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. *The EMBO Journal*, **21**, 1360–1368.

De Veylder, L., Beeckman, T. & Inzé, D. (2007) The ins and outs of the plant cell cycle. *Nature Reviews in Molecular Cell Biology*, **8**, 655–665.

del Pozo, J.C., Diaz-Trivino, S., Cisneros, N. & Gutierrez, C. (2006) The balance between cell division and endoreplication depends on E2FCDPB, transcription factors regulated by the ubiquitin- SCFSKP2A pathway in Arabidopsis. *The Plant Cell*, **18**, 2224–2235.

Dotto, M. & Casati, P. (2017) Developmental reprogramming by UV-B radiation in plants. *Plant Science*, **267**, 96–101.

Fierro, A.C., Leroux, O., De Coninck, B., Cammue, B.P.A., Marchal, K., Prinsen, E. et al. (2015) Ultraviolet-B radiation stimulates downward leaf curling in Arabidopsis thaliana. *Plant Physiology and Biochemistry*, **93**, 9–17.

Fina, J.P., Casadevall, R., AbdElgawad, H., Prinsen, E., Markakis, M., Beemster, G. et al. (2017) UV-B inhibits leaf growth through changes in Growth Regulating Factors and gibberellin levels. *Plant Physiology*, **174**, 1110–1126.

Friedberg, E.C., Walker, G.C. & Siede, W. (1995) *DNA damage*. Washington, DC: ASM Press.

Fulcher, N. & Sablowski, R. (2009) Hypersensitivity to DNA damage in plant stem cell niches. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 20984–20988. https://doi.org/10.1073/pnas.0909218106

Furukawa, T., Curtis, M.J., Tominey, C.M., Duong, Y.H., Wilcox, B.W.L., Aggoune, D. et al. (2010) A shared DNA-damage-response pathway for induction of stem-cell death by UVB and by gamma irradiation. *DNA Repair (Amst)*, **9**, 940–948.

Galbraith, D.W., Harkins, K.R. & Knapp, S. (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. *Plant Physiology*, **96**, 985–989.

Gómez, M.S., Falcone Ferreyra, M.L., Sheridan, M.L. & Casati, P. (2019) Arabidopsis E2Fc is required for the DNA damage response under UV-B radiation epistatically over the microRNA396 and independently of E2Fe. *The Plant Journal*, **97**, 749–764.

Harashima, H. & Sugimoto, K. (2016) Integration of developmental and environmental signals into cell proliferation and differentiation through RETINOBLASTOMA-RELATED 1. *Current Opinion in Plant Biology*, **29**, 95–103.

Hectors, K., Jacques, E., Prinsen, E., Guisez, Y., Verbelen, J.P., Jansen, M.A.K. et al. (2010) UV radiation reduces epidermal cell expansion in leaves of Arabidopsis thaliana. *Journal of Experimental Botany*, **61**, 4339–4349.

Heyman, J., Van den Daele, H., De Wit, K., Boudolf, V., Berckmans, B., Verkest, A. et al. (2011) The Arabidopsis Ultraviolet-B-Insensitive4 maintains cell division activity by temporal inhibition of the anaphase-promoting complex/cyclosome. *The Plant Cell*, **23**, 4394–4410.

Horiguchi, G., Kim, G.T. & Tsukaya, H. (2005) The transcription factor AtGRF5 and the transcription coactivator AN3 regulate cell proliferation in leaf primordia of *Arabidopsis thaliana*. *The Plant Journal*, **43**, 68–78.

Horvath, B.M., Kourova, H., Nagy, S., Nemeth, E., Magyar, Z., Papdi, C. et al. (2017) Arabidopsis RETINOBLASTOMA RELATED directly regulates DNA damage responses through functions beyond cell cycle control. *The EMBO Journal*, **36**, 1261–1278.

Klepikova, A.V., Kasianov, A.S., Gerasimov, E.S., Logacheva, M.D. & Penin, A.A. (2016) A high resolution map of the Arabidopsis thaliana developmental transcriptome based on RNA-seq profiling. *The Plant Journal*, **88**, 1058–1070.

Kobayashi, K., Suzuki, T., Iwata, E., Nakamichi, N., Suzuki, T., Chen, P. et al. (2015) Transcriptional repression by MYB3R proteins regulates plant organ growth. *The EMBO Journal*, **34**, 1992–2007.

Lammens, T., Boudolf, V., Kheibarshekan, L., Zalmas, L.P., Gaamouche, T., Maes, S. et al. (2008) Atypical E2F activity restrains APC/$C^{CCS52A2}$ function obligatory for endocycle onset. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 14721–14726.

Leviczky, T., Molnár, E., Papdi, C., Öszi, E., Horváth, G.V., Vizler, C. et al. (2019) E2FA and E2FB transcription factors coordinate cell proliferation with seed maturation. *Development*, **146**, 179333.

Li, X., Cai, W., Liu, Y., Li, H., Fu, L., Liu, Z. et al. (2017) Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, 2765–2770.

Magyar, Z., Atanassova, A., De Veylder, L., Rombauts, S. & Inzé, D. (2000) Characterization of two distinct DP-related genes from *Arabidopsis thaliana*. *FEBS Letters*, **486**, 79–87.

Magyar, Z., Bögre, L. & Ito, M. (2016) DREAMs make plant cells to cycle or to become quiescent. *Current Opinion in Plant Biology*, **34**, 100–106.

Magyar, Z., De Veylder, L., Atanassova, A., Bakó, L., Inzé, D. & Bögre, L. (2005) The role of the Arabidopsis E2FB transcription factor in regulating auxin-dependent cell division. *The Plant Cell*, **17**, 2527–2541.

Magyar, Z., Horváth, B., Khan, S., Mohammed, B., Henriques, R., De Veylder, L. et al. (2012) Arabidopsis E2FA stimulates proliferation and endocycle separately through RBR-bound and RBR-free complexes. *The EMBO Journal*, **31**, 1480–1493.

Mariconti, L., Pellegrini, B., Cantoni, R., Stevens, R., Bergounioux, C., Cella, R. et al. (2002) The E2F family of transcription factors from Arabidopsis thaliana. Novel and conserved components of the retinoblastoma/E2F pathway in plants. *Journal of Biological Chemistry*, **277**, 9911–9919.

Maulión, E., Gómez, M.S., Falcone Ferreyra, M.L., Bustamante, C.A. & Casati, P. (2019) AtCAF-1 mutants show different DNA damage responses after ultraviolet-B than those activated by other genotoxic agents in leaves". *Plant Cell and Environment*, **42**, 2730–2745.

Menges, M., De Jager, S.M., Gruissem, W. & Murray, J.A.H. (2005) Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. *The Plant Journal*, **41**, 546–566.

Naouar, N., Vandepoele, K., Lammens, T., Casneuf, T., Zeller, G., Van Hummelen, P. et al. (2009) Quantitative RNA expression analysis with Affymetrix Tiling 1.0R arrays identifies new E2F target genes. *The Plant Journal*, **57**, 184–194.

Oszi, E., Papdi, C., Mohammed, B., Petkó-Szandtner, A., Leviczky, T., Molnár, E. et al. (2020) E2FB interacts with RETINOBLASTOMA RELATED and regulates cell proliferation during leaf development. *Plant Physiology*, **182**, 518–533.

Radziejwoski, A., Vlieghe, K., Lammens, T., Berckmans, B., Maes, S., Jansen, M.A.K. et al. (2011) Atypical E2F activity coordinates PHR1 photolyase gene transcription with endoreduplication onset. *The EMBO Journal*, **30**, 355–363.

Ramirez-Parra, E., Frundt, C. & Gutierrez, C. (2003) A genome-wide identification of E2F regulated genes in Arabidopsis. *The Plant Journal*, **33**, 801–811.

Ramirez-Parra, E. & Gutierrez, C. (2007) The many faces of chromatin assembly factor 1. *Trends in Plant Science*, **12**, 570–576.

Rozen, S. & Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S.A. & Misener, S. (Eds.) *Bioinformatics methods and protocols: Methods in molecular biology*. Totowa NJ: Humana Press, pp. 365–386.

Sadasivam, S. & DeCaprio, J.A. (2013) The DREAM complex: master coordinator of cell cycle-dependent gene expression. *Nature Reviews Cancer*, **13**, 585–595.

Sozzani, R., Maggio, C., Varotto, S., Canova, S., Bergounioux, C., Albani, D. et al. (2006) Interplay between Arabidopsis activating factors E2Fb and E2Fa in cell cycle progression and development. *Plant Physiology*, **140**, 1355–1366.

van den Heuvel, S. & Dyson, N.J. (2008) Conserved functions of the pRB and E2F families. *Nature Reviews Molecular Cell Biology*, **9**, 713–724.

Vandepoele, K., Vlieghe, K., Florquin, K., Hennig, L., Beemster, G.T., Gruissem, W. et al. (2005) Genome-wide identification of potential plant E2F target genes. *Plant Physiology*, **139**, 316–328.

Vlieghe, K., Boudolf, V., Beemster, G.T.S., Maes, S., Magyar, Z., Atanassova, A. et al. (2005) The DP-E2F-like gene DEL1 controls the endocycle in Arabidopsis thaliana. *Current Biology*, **15**, 59–63.

Wargent, J.J., Gegas, V.C., Jenkins, G.I., Doonan, J.H. & Paul, N.D. (2009a) UVR8 in Arabidopsis thaliana regulates multiple aspects of cellular differentiation during leaf development in response to ultraviolet B radiation. *New Phytology*, **183**, 315–326.

Wargent, J.J., Moore, J.P., Roland Ennos, A. & Paul, N.D. (2009b) Ultraviolet radiation as a limiting factor in leaf expansion and development. *Photochemistry and Photobiology*, **85**, 279–286.