

Repression of Growth Regulating Factors by the MicroRNA396 Inhibits Cell Proliferation by UV-B Radiation in *Arabidopsis* Leaves^{CW}

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Because of their sessile lifestyle, plants are continuously exposed to solar UV-B radiation. Inhibition of leaf growth is one of the most consistent responses of plants upon exposure to UV-B radiation. In this work, we investigated the role of *GROWTH-REGULATING FACTORS* (*GRFs*) and of microRNA miR396 in UV-B-mediated inhibition of leaf growth in *Arabidopsis thaliana* plants. We demonstrate that miR396 is upregulated by UV-B radiation in proliferating tissues and that this induction is correlated with a decrease in *GRF1*, *GRF2*, and *GRF3* transcripts. Induction of miR396 results in inhibition of cell proliferation, and this outcome is independent of the UV-B photoreceptor UV resistance locus 8, as well as ATM AND RAD3-RELATED and the mitogen-activated protein kinase MPK6, but is dependent on MPK3. Transgenic plants expressing an artificial target mimic directed against miR396 (*MIM396*) with a decrease in the endogenous microRNA activity or plants expressing miR396-resistant copies of several *GRFs* are less sensitive to this inhibition. Consequently, at intensities that can induce DNA damage in *Arabidopsis* plants, UV-B radiation limits leaf growth by inhibiting cell division in proliferating tissues, a process mediated by miR396 and *GRFs*.

INTRODUCTION

Because of their sessile lifestyle, plants have evolved adaptations to diverse environmental factors, including solar radiation. The solar spectrum includes light qualities that are essential for photosynthesis, but certain wavelengths can damage cells. UV-B radiation (280 to 315 nm) reaching the earth induces a broad range of physiological responses. Plants have UV-B-specific photoregulatory responses (Ulm, 2003). In *Arabidopsis thaliana*, these photomorphogenic responses are regulated by the recently identified UV-B photoreceptor UV RESISTANCE LOCUS8 (*UVR8*), which was shown to enhance survival under simulated sunlight with realistic UV-B radiation levels (Favory et al., 2009; Rizzini et al., 2011). *UVR8*, in cooperation with CONSTITUTIVE PHOTOMORPHOGENESIS1, is required for UV-B-mediated upregulation of the gene encoding the HY5 transcription factor (Ulm et al., 2004; Brown et al., 2005). *UVR8* and CONSTITUTIVE PHOTOMORPHOGENESIS1 interact in the nucleus after UV-B exposure (Oravec et al., 2006), and this very early step in UV-B signaling is proposed to initiate UV-B acclimation responses (Favory et al., 2009).

At high intensities, UV-B radiation induces mostly unspecific damage responses in organisms, and plants contain damage

signaling pathways that are partially conserved among other organisms (Culligan et al., 2004; Cools and De Veylder, 2009; González Besteiro et al., 2011). To cope with UV-B-damaged DNA, the protein kinase ATM AND RAD3-RELATED (*ATR*) serves as a key regulator of the DNA damage response; in *Arabidopsis*, *atr* mutants are hypersensitive to UV-B radiation (Culligan et al., 2004). Apart from the *ATR*-mediated pathway, mitogen-activated protein kinase (*MAPK*) signaling is another important UV radiation response pathway (González Besteiro et al., 2011; González Besteiro and Ulm, 2013). In *Arabidopsis*, mitogen-activated protein kinases *MPK3* and *MPK6* are activated upon exposure to DNA-damaging agents, and the *MPK3/MPK6* cascade has been suggested to be a major pathway contributing to UV-B-induced DNA damage (González Besteiro and Ulm, 2013). *MKP1* negatively regulates *MAPK* signaling by dephosphorylating the stress-activated *MPK3* and *MPK6* (Bartels et al., 2009, 2010). Therefore, *mkp1* hypersensitivity to UV-B stress is associated with hyperactive *MPK3* and *MPK6* (González Besteiro et al., 2011). Moreover, *MKP1* has been suggested to function as a trigger of cell cycle checkpoints (Cools and De Veylder, 2009; González Besteiro et al., 2011).

Inhibition of leaf growth is one of the most consistent plant responses to solar UV-B exposure, and this radiation, both in the field and in controlled environments, has been shown to consistently limit leaf growth in a wide range of species (Ballaré et al., 2001; Searles et al., 2001; Flint et al., 2003). In *Arabidopsis*, leaf size goes through an initial phase of cell proliferation, followed by a period of cell expansion (Donnelly et al., 1999; Piazza et al., 2005; Tsukaya, 2005; Andriankaja et al., 2012). UV-B-irradiated plants show less elongated leaves, stems, and hypocotyls (Jansen, 2002). However, available data about the effects of UV-B radiation on cell proliferation and cell

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expansion are contradictory, probably reflecting differences in experimental conditions. Acute, stress-inducing UV-B conditions cause necrosis and inhibit cell proliferation, while lower doses and/or chronic UV-B treatments affect both cell proliferation and expansion (Staxen and Bornman, 1994; Nogués et al., 1998; Laakso et al., 2000; Hopkins et al., 2002; Hofmann et al., 2003; Kakani et al., 2003; Hectors et al., 2007, 2010; Wargent et al., 2009a; Robson and Aphalo, 2012). For example, fewer epidermal cells were observed after UV-B exposure in some species (Gonzalez et al., 1998; Hopkins et al., 2002); however, stimulation of cell proliferation by UV-B radiation has also been reported in petunia (*Petunia hybrida*; Staxen and Bornman, 1994), and increases in leaf thickness attributed to increases in the number of parenchyma cells were reported in *Brassica carinata* and *Medicago sativa* (Bornman and Vogelmann, 1991). In lettuce (*Lactuca sativa*) leaves, UV-B radiation reduced the leaf expansion rate and final leaf size (Wargent et al., 2009a). In *Arabidopsis*, UV-induced morphological changes in acclimated plants include decreased rosette diameter, decreased inflorescence height, and increased numbers of flowering stems; these phenotypes have been interpreted as a redistribution rather than a cessation of growth during chronic UV-B radiation treatments (Hectors et al., 2007). Despite the diverse responses reported, the underlying mechanisms of the reduction in leaf growth under UV-B stress remains poorly understood.

Arabidopsis GROWTH-REGULATING FACTOR (GRF) proteins are a family comprising nine members; they regulate various developmental processes in leaves and cotyledons in a functionally redundant manner (Kim et al., 2003). GRF genes occur in the genomes of all seed plants analyzed to date (van der Knaap et al., 2000; Kim et al., 2003; Choi et al., 2004; Zhang et al., 2011). Transgenic plants overexpressing *GRF1* or *GRF2* have larger leaves than wild-type plants, whereas triple null mutants of *grf1_2_3* have smaller and narrower leaves as well as fused cotyledons (Kim et al., 2003). The GRF proteins contain the conserved Glu-Leu-Glu and Trp-Arg-Cys domains in their N-terminal regions, which define the GRF family. The Trp-Arg-Cys domain has a functional nuclear localization signal and a DNA binding motif consisting of the conserved spacing of three Cys and one His residues. The Glu-Leu-Glu domain interacts with the N-terminal domain of any of the three GRF-INTERACTING FACTORS (GIFs) in *Arabidopsis* (Kim and Kende, 2004). Like *grf* mutants, a *gif1* mutant develops narrower leaves and petals than do wild-type plants, and combinations of *gif1* and *grf* mutations show a cooperative effect (Kim and Kende, 2004). The narrow leaf phenotype of *gif1*, as well as that of the *grf* triple mutant, reflects a reduction in cell number (Kim and Kende, 2004). Thus, GRFs and GIFs are part of a complex pathway involved in regulating cellular proliferation and, hence, the growth and shape of leaves.

Several GRF levels are regulated posttranscriptionally by the microRNA miR396. In leaf primordia, miR396 is expressed at low levels and increases during organ development, while *GRFs* show the opposite pattern (Rodriguez et al., 2010; Debernardi et al., 2012). miR396 accumulates preferentially in the distal part of young developing leaves and attenuates cell proliferation through the repression of *GRF* activity and cell cycle genes, fine-tuning the final number of cells in leaves (Rodriguez et al., 2010).

Many microRNAs (miRNAs) have been predicted, and some have been confirmed experimentally to be involved in a variety of abiotic stress responses (Lu and Huang, 2008). In particular, miR396 is regulated by high salinity, drought, and low and high temperature in *Arabidopsis* and in other plant species, sometimes showing opposite regulation depending on the species (Liu et al., 2008; Shen et al., 2010; Zhou et al., 2010; Kantar et al., 2011; Giacomelli et al., 2012). Here, we investigated the role of GRFs and miR396 in the inhibition of leaf growth provoked by UV-B radiation in *Arabidopsis*. miR396 is upregulated by UV-B radiation in proliferating tissues, and this induction is correlated with a decrease in *GRF1*, *GRF2*, and *GRF3* transcripts. The induction of miR396 causes an inhibition of cell proliferation, but not of cell expansion, in developing leaves with proliferating cells, and its function is independent of the UV-B photoreceptor UVR8 as well as ATR and MPK6, but is dependent on MKP3. Further support for a direct connection between miR396 and the regulation of growth during UV-B responses was obtained by reducing the effectiveness of this miRNA in transgenic plants expressing an artificial target with a motif that can be recognized by miR396, but produces a noncleavable transcript that sequesters the miRNA, thus reducing the active level of miR396 (target mimic, *MIM396*) or expressing miR396-resistant *GRFs*.

RESULTS

UV-B Radiation Affects Cell Division but Not Cell Expansion in Proliferating Leaves and Modulates the Expression of miR396, *GRF1*, *GRF2*, and *GRF3* in *Arabidopsis*

One of the primary effects of UV-B radiation on plants is reduction in biomass (Bornman and Teramura, 1993); however, the underlying process that is affected depends on the species and treatments employed. Thus, we first investigated the effect of a single UV-B treatment for 4 h with an intensity of 2 Wm^{-2} in the rosette area of Columbia-0 (Col-0) *Arabidopsis* plants. These UV-B levels are found in many places on Earth and, for example, are similar to the average UV-B intensity of noon solar radiation in summer in Rosario, Argentina. Plants were irradiated with a single UV-B light treatment 14 d after sowing (DAS). At this stage, leaf #5 has proliferating cells, as shown by the expression of *Cyclin B1;1* (*CYCB1;1*):*GUS* (for β -glucuronidase), a reporter of a mitotic cells (see Supplemental Figure 1 online; Donnelly et al., 1999). Figures 1A and 1B show that, 4 d after the end of this treatment, the rosette area is significantly smaller ($P < 0.05$) in UV-B-treated plants than in plants grown under control illumination, and the difference in rosette size between treated and untreated plants persists 10 d after the treatment.

Then, we measured the final leaf area and the average palisade parenchyma cell area and calculated the number of palisade cells in fully expanded leaf #5. As shown in Figure 1C, while the leaf #5 area is significantly reduced in UV-B-treated plants, the average cell area is similar in treated and untreated leaves (Figures 1D and 1F). Thus, after this treatment and in developing leaves with proliferating cells, UV-B radiation inhibits cell proliferation, and treated leaves have fewer cells than control leaves from untreated plants (Figure 1E).

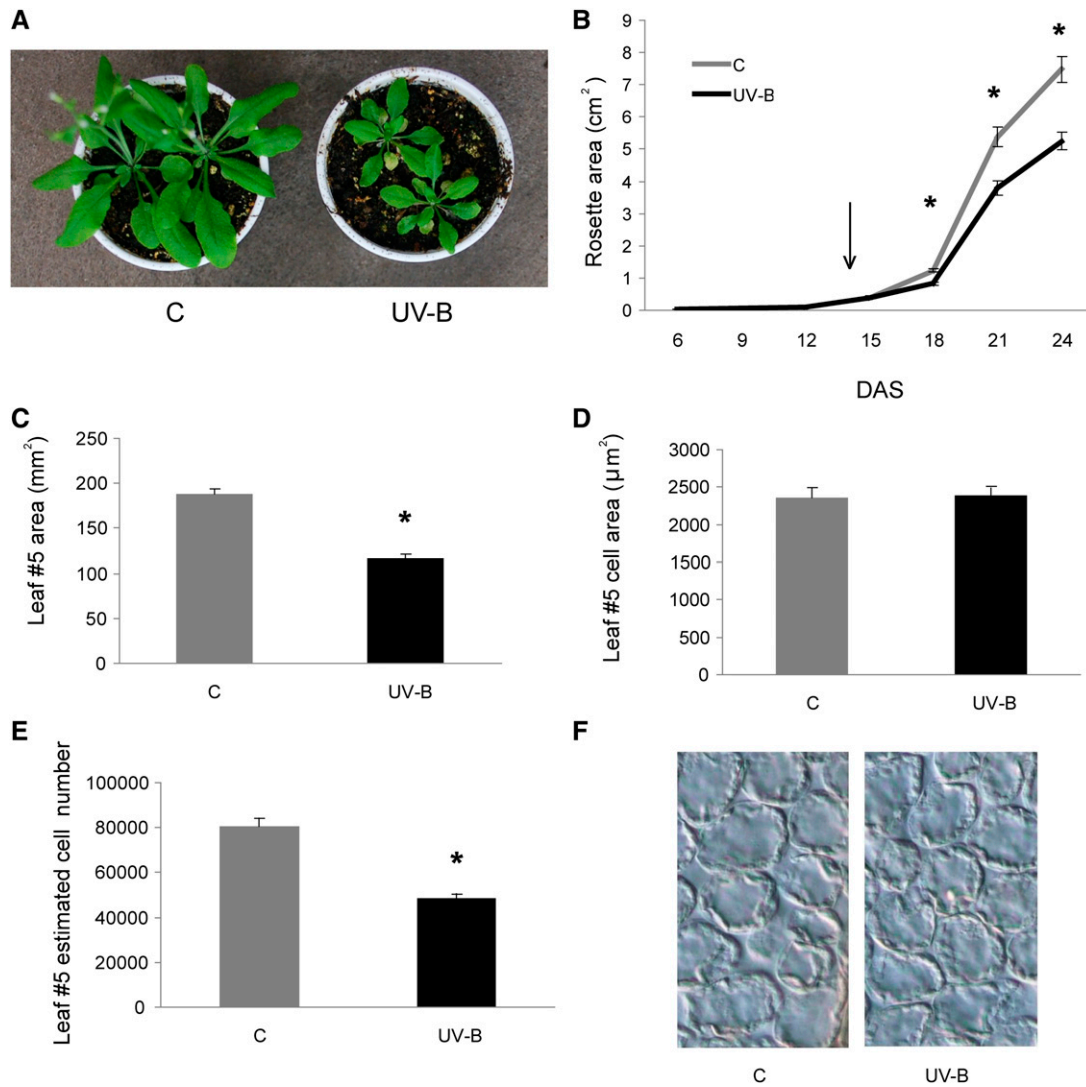


Figure 1. UV-B Affects Cell Proliferation but Not Cell Expansion in Developing Leaves of Col-0 *Arabidopsis* Plants with Proliferating Cells.

(A) Col-0 plants treated with UV-B radiation for 4 h (2 Wm^{-2} ; right) or kept under control (C) conditions in the absence of UV-B radiation at 14 DAS, 15 d after the end of the treatment.

(B) Rosette area of control and UV-B-treated plants measured every 3 d from germination until day 24. Plants were UV-B treated at 14 DAS (indicated with an arrow).

(C) to (E) Average leaf area (C), cell area (D), and estimated cell number (E) of fully expanded leaf #5 from UV-B-treated or control Col-0 *Arabidopsis* plants. Results represent the average of six biological replicates \pm SE. Asterisks indicate significant differences as determined by Student's *t* test ($P < 0.05$).

(F) Paradermal view of palisade parenchyma cells from UV-B-treated and control leaf # 5. [See online article for color version of this figure.]

Effects of UV-B Irradiation on miR396 and *GRF* Levels

Interestingly, the smaller *Arabidopsis* plants treated with UV radiation resemble transgenic plants overexpressing miRNA miR396, which also have fewer cells per leaf (Rodriguez et al., 2010). miR396 represses GRF transcription factors, which in turn regulate leaf development by promoting cell proliferation. Therefore, we investigated if the inhibition of cell proliferation by UV-B radiation involves a premature induction of miR396 in

leaves in the proliferative phase. First, we measured the expression levels of several *GRF* transcription factors in leaf #5 from UV-B-treated and untreated *Arabidopsis* plants. The samples were collected immediately after the end of the 4 h UV-B treatment. Quantitative RT-PCR (qRT-PCR) results show that *GRF1*, *GRF2*, and *GRF3* are significantly downregulated by UV-B radiation in this actively dividing organ, while *GRF6* and *GRF9* levels are not changed by the treatment (Figure 2A). It is interesting to note that while *GRF1*, *GRF2*, *GRF3*, and *GRF9* are

regulated posttranscriptionally by miR396, *GRF6* is not (Rodriguez et al., 2010).

Next, we investigated the expression of miR396 in UV-B-treated and control leaf #5. Both small RNA gel blot (Figure 2B) and qRT-PCR analyses (Figure 2C) demonstrated that miR396 is induced by UV-B radiation. Thus, the decrease in *GRF1*, *GRF2*, and *GRF3* levels may be, at least in part, due to the negative regulation by miR396 after the UV-B treatment, and the decreased levels of some *GRF* transcripts may be the cause of the inhibition of cell proliferation measured in our experiments.

To test if the increase in miR396 levels by UV-B radiation is specific for leaf #5 at a particular developmental stage, or if it is a general response to UV-B radiation in different organs where this miRNA is expressed, we investigated its UV-B regulation at different developmental times and organ types. Supplemental Figure 2 online shows that miR396 is upregulated by UV-B radiation in leaves and apices, as well as in pools of leaves of different ages.

We then analyzed the kinetics of miR396 accumulation by UV-B radiation. To address this point, a different UV-B treatment was applied. Plants were exposed to UV-B radiation at the same intensity used for experiments shown in Figures 1 and 2 for 2 h

each day during four consecutive days. Leaf #5 samples were collected immediately after the end of the treatment after 1 and 4 d of UV-B exposure. This UV-B treatment also inhibits cell proliferation in leaf #5 (see Supplemental Figures 3A to 3D online). Supplemental Figure 3F online shows that miR396 is induced 2.7-fold by UV-B radiation after 2 h of irradiation compared with the untreated leaves. This induction is significant, although lower than the induction measured after 4 h of UV-B treatment (fourfold; Figure 2C). However, after 4 d of irradiation, miR396 levels are similar in both UV-B-treated and control plants, while GRFs are still repressed by UV-B radiation (see Supplemental Figure 3E online). Therefore, we suggest that miR396 may act as an early and transient signal to inhibit cell division by UV-B radiation, but at longer exposure times, repression of *GRF* expression may be mainly transcriptionally regulated.

UV-B Irradiation Inhibits Cell Proliferation through the Activation of miR396

To evaluate if the effect of UV-B radiation in cell proliferation is mediated by miR396, we analyzed the effect of UV-B radiation

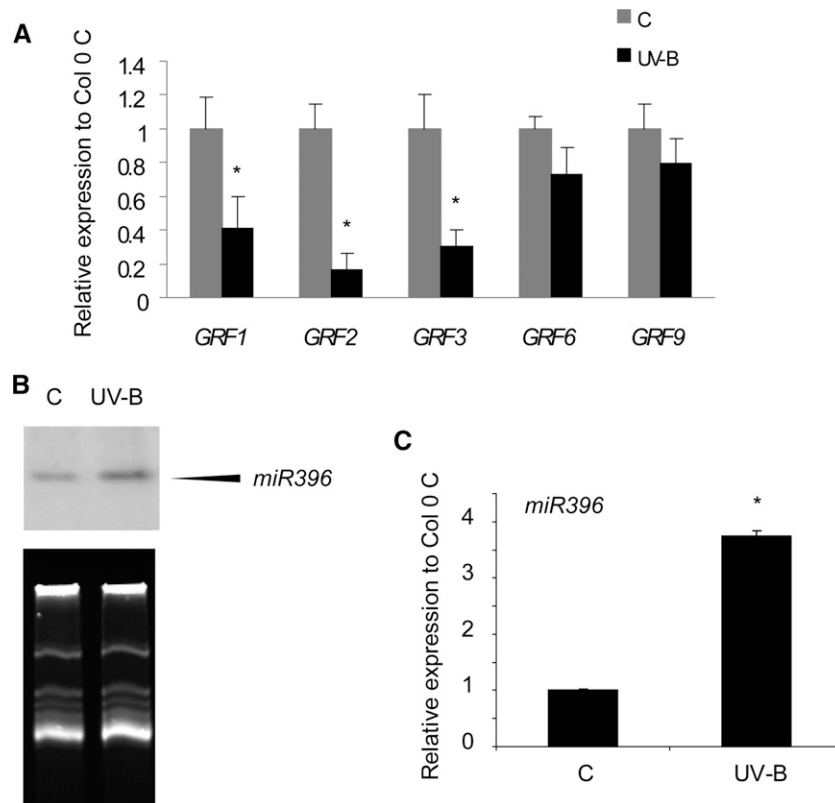


Figure 2. UV-B Effect on Expression Levels of *GRFs* and miR396 in *Arabidopsis* Leaf #5.

(A) Expression levels relative to Col-0 under control conditions (C) of *GRF1*, *GRF2*, *GRF3*, *GRF6*, and *GRF9* genes determined by qRT-PCR analysis in Col-0 leaf #5 under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} (UV-B).

(B) Small RNA gel blot showing the expression of miR396 in leaf #5 under control conditions in the absence of UV-B (C) or immediately after a 4-h-UV-B treatment at 2 Wm^{-2} (UV-B).

(C) Expression levels relative to Col-0 miR396 in leaf #5 under control conditions in the absence of UV-B (C) or immediately after a 4-h-UV-B treatment at 2 Wm^{-2} (UV-B) as determined by qRT-PCR. Results represent the average of six biological replicates \pm se. Asterisks indicate the statistical differences applying the Student's *t* test ($P < 0.05$).

on growth inhibition using transgenic *Arabidopsis* plants expressing an artificial target mimic directed against miR396 (*MIM396*). Transgenic plants harboring *35S:MIM396* have a decrease in endogenous miRNA activity (Debernardi et al., 2012), as expected from the target mimicry technology (Franco-Zorrilla et al., 2007; Todesco et al., 2010). Under normal conditions, transgenic plants harboring the miR396 target mimic look similar to wild-type Col-0 plants (Figures 3A and 3B). However,

transgenic plants treated with UV-B radiation for 4 h have a bigger rosette than Col-0 plants (Figures 3A and 3C). In *MIM396* UV-B-irradiated plants, average leaf #5 area is larger than that of Col-0 plants (Figure 3D), and this is because *MIM396* leaf #5 have more cells than Col-0 leaves, while the average cell area remains similar (Figures 3E and 3F). In addition, leaf #5 from *MIM396* plants has lower levels of miR396 than those of Col-0 plants. Although miR396 is still UV-B regulated in

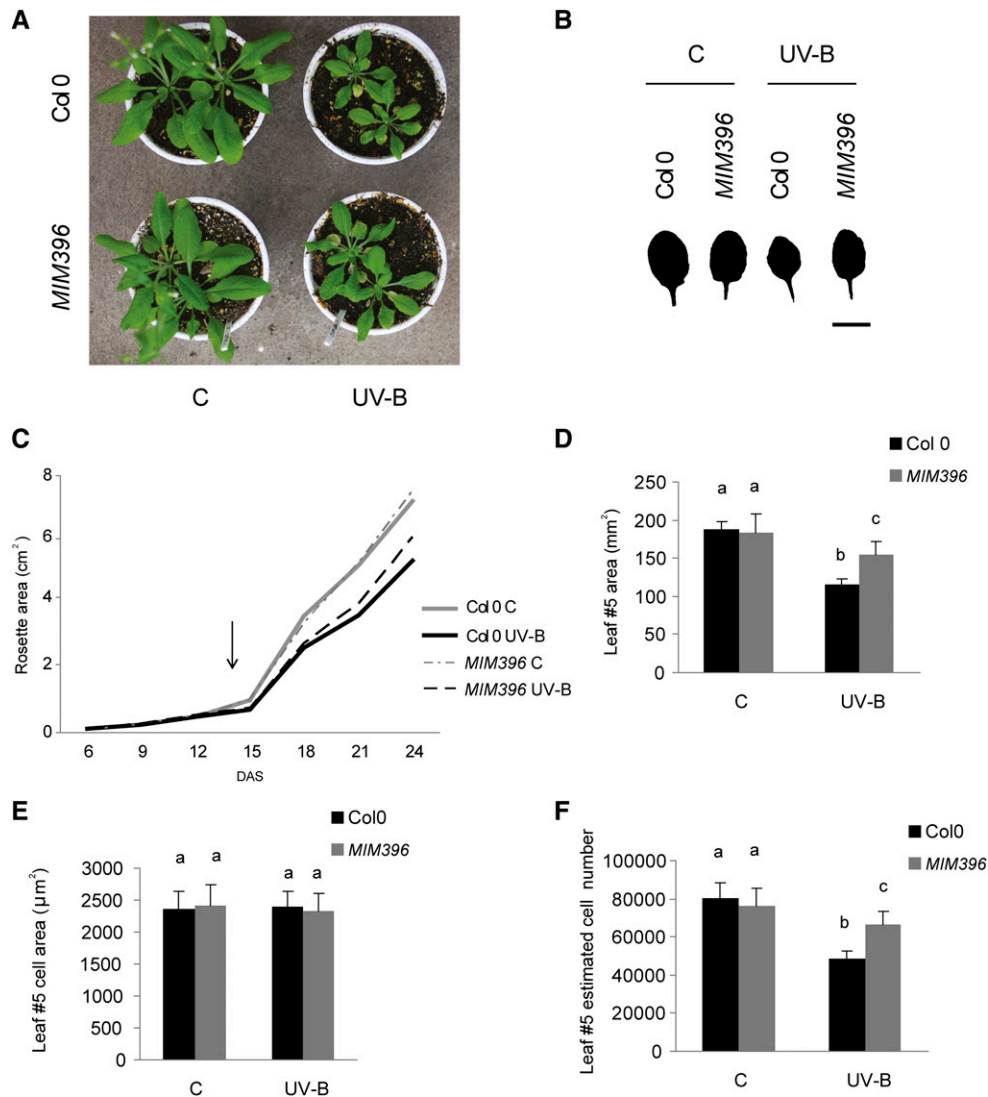


Figure 3. UV-B Repression of Cell Proliferation Is Mediated by miR396.

(A) Col-0 and plants expressing an artificial target mimic directed against miR396 (*MIM396*) that were treated with UV-B radiation for 4 h (2 Wm^{-2} ; right) or were kept under conditions in the absence of UV-B, 15 d after the end of the treatment. C, control.

(B) Silhouettes of leaf #5 detached from control and UV-B plants shown in (A). Bar = 1 cm.

(C) Rosette areas of control and UV-B-treated Col-0 and *MIM396* plants measured every 3 d from germination until day 24. Plants were UV-B treated at 14 DAS (indicated with an arrow).

(D) to (F) Average leaf area (D), cell area (E), and estimated cell number (F) of fully expanded leaf #5 from UV-B-treated versus control Col-0 and *MIM396* *Arabidopsis* plants. Results represent the average of 10 biological replicates \pm SE. Different letters indicate statistically significant differences applying ANOVA test ($P < 0.05$).

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the transgenic plants, its levels remained significantly lower than those observed in Col-0 leaves (Figure 4A).

While *GRF1* to *GRF3* were strongly downregulated in Col-0 plants under UV-B irradiation, we observed that they were marginally affected by a similar treatment in *MIM396* plants. Thus, the repression of the GRFs after the UV-B treatment is at least partially achieved by the induction of miR396 in Col-0 plants. Altogether, our results highlight the importance of the activation of miR396 in the inhibition of cell proliferation in young proliferating leaves of *Arabidopsis* under UV-B irradiation.

The Inhibition of Cell Proliferation by UV-B Light Requires the Activity of GRFs

In our experiments, *GRF3* is expressed in leaf #5, which contains actively dividing cells at the moment of the treatment, and it also shows a significant decrease in its mRNA levels by UV-B radiation in this organ (Figure 2). Therefore, we investigated if its regulation through miR396 is important for cell proliferation inhibition by UV-B radiation. For this purpose, we analyzed the

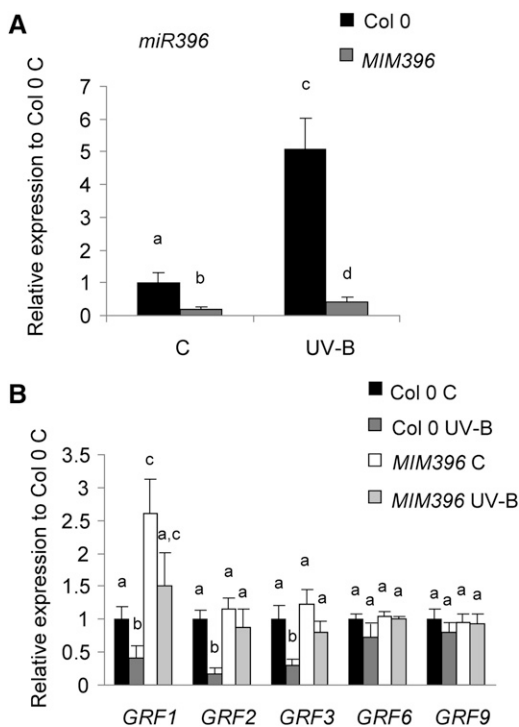


Figure 4. UV-B Effect on Expression Levels of *GRFs* and miR396 in Leaf #5 of Col-0 and *MIM396* Plants.

(A) Expression levels relative to Col-0 control (C) of miR396 in leaf #5 of Col-0 and *MIM396* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} determined by qRT-PCR.

(B) Expression levels relative to Col-0 control of *GRF1*, *GRF2*, *GRF3*, *GRF6*, and *GRF9* determined by qRT-PCR in Col-0 and *MIM396* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} (UV-B). Results represent the average of six biological replicates \pm SE. Different letters indicate statistically significant differences applying ANOVA test ($P < 0.05$).

effect of UV-B radiation in cell proliferation in transgenic plants expressing a miR396-resistant version of *GRF3* under its own promoter (*rGRF3*). This transgene was prepared by introducing synonymous mutations in the miR396 binding site of *GRF3* in a similar way to previous work performed on *GRF2* (Rodríguez et al., 2010).

Next, we tested whether the UV-B treatment affected plants harboring an *rGRF3* transgene. As expected from an increase in *GRF* levels, *rGRF3* plants have bigger leaves than Col-0 (Figure 5A; see Supplemental Figure 4 online). Therefore, we measured the relative effect of the UV-B stress on their leaves. After the UV-B treatment, even if these plants show a decrease in the rosette and leaf area, the relative decrease was significantly lower than that measured in Col-0 plants (Figures 5C and 5D; see Supplemental Figure 4 online). This latter change was also a consequence of an inhibition of cell proliferation (Figure 5F), as leaf cell size is not affected by the treatment (Figure 5E), as measured in Col-0 plants. Therefore, miR396 regulation of cell proliferation by UV-B radiation is through the repression of *GRF* transcription factors in *Arabidopsis*.

qRT-PCR experiments were done to analyze if miR396 is induced by UV-B radiation in the *rGRF3* plants. Figure 6A shows that expression of a miR396-resistant copy of *GRF3* in transgenic plants does not affect the expression of miR396 or its UV-B-induced upregulation. In addition, transcript levels of *GRFs* other than *GRF3* after irradiation with UV-B, or under control conditions, are not changed in these transgenic plants either (Figure 6B). It is interesting to note that, although *GRF3* transcript levels are significantly higher in the transgenic plants than in Col-0 plants, UV-B radiation still reduces the expression of *GRF3* in the transgenic plants (Figure 6B). Thus, UV-B regulation of *GRFs* is probably not only posttranscriptional, but also transcriptional, as also shown in Supplemental Figure 3E online.

To further investigate the role of other *GRFs* in cell proliferation during UV-B exposure, experiments were repeated using the miR396-resistant version of *GRF2* under its own promoter (*rGRF2*; Rodríguez et al., 2010). Similarly as measured for *rGRF3* plants, the decrease in leaf #5 area by UV-B radiation in the *rGRF2* plants is lower than that of the Col-0 plants, and this is also due to a lower inhibition of cell proliferation and not due to a difference in cell expansion (see Supplemental Figure 5 online). Therefore, the role of *GRFs* in cell proliferation during UV-B treatment is redundant, at least for *GRF2* and *GRF3*.

Inhibition of Cell Proliferation by UV-B Radiation Mediated by miR396 and GRFs Is Not Mediated by the UV-B Photoreceptor UVR8, ATR, or MPK6 but Requires MPK3

As described in the Introduction, some UV-B responses, in particular those elicited by low-fluence UV-B radiation, are regulated by UVR8, a recently identified UV-B photoreceptor (Rizzini et al., 2011). However, there are some responses to higher UV-B intensities that are independent of this photoreceptor, particularly DNA damage repair responses, which can induce cell cycle arrest (Ulm, 2003). Thus, to investigate if miR396-mediated cell proliferation inhibition is dependent on UVR8, the UV-B effect in plant growth was analyzed using *uvr8* knockout mutant plants (Brown et al., 2005). Under our

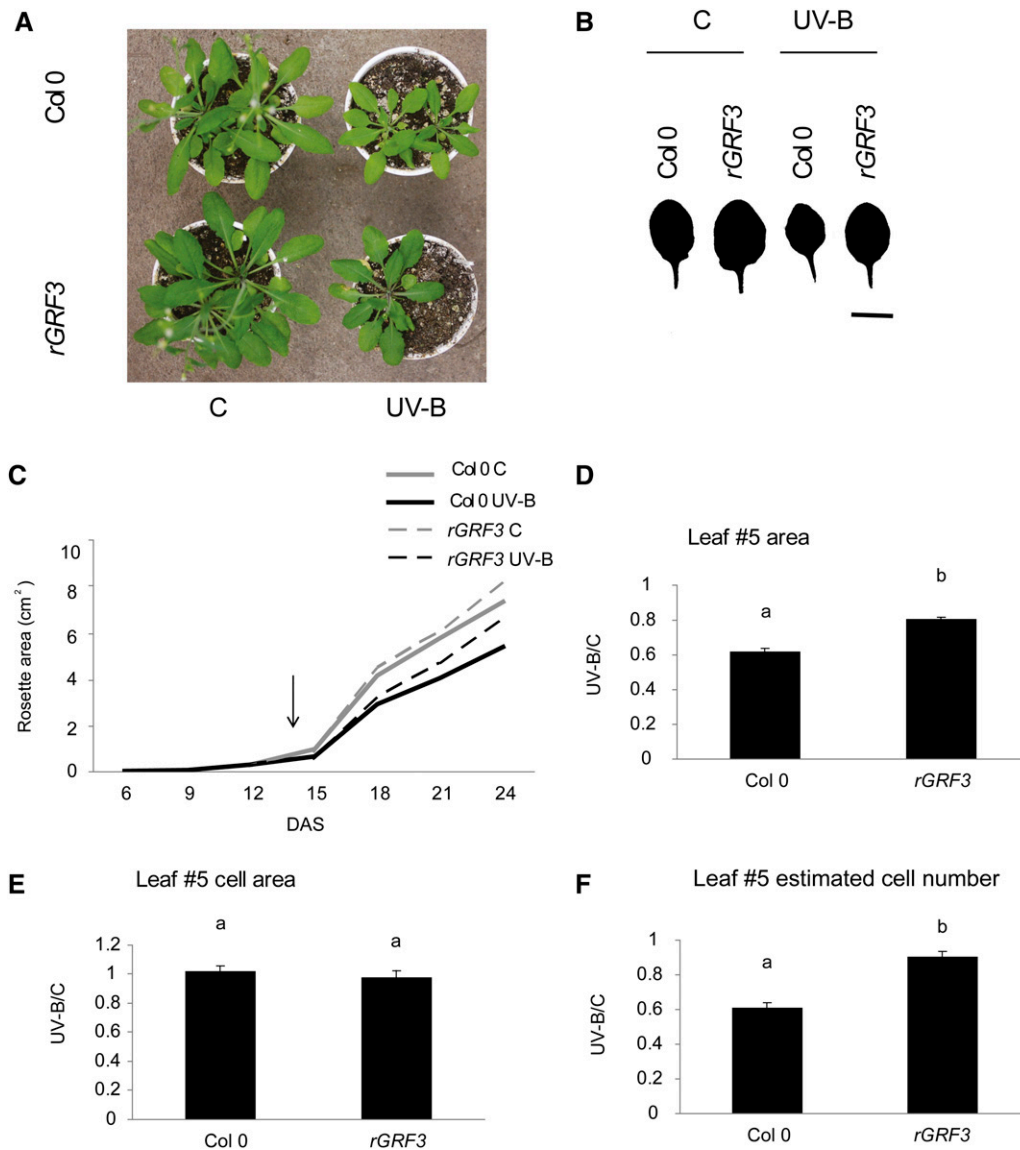


Figure 5. UV-B Effect in Cell Proliferation Is Mediated by miR396-Induced Repression of GRFs.

(A) Col-0 and plants expressing an miR396-resistant copy of *GRF3* (*rGRF3*) that were treated with UV-B radiation for 4 h (2 Wm^{-2} ; right) or were kept under conditions in the absence of UV-B (C), 15 d after the end of the treatment.

(B) Leaf #5 silhouettes from control and UV-B plants shown in (A). Bar = 1 cm.

(C) Rosette area of control and UV-B treated Col-0 and *rGRF3* plants measured every 3 days from germination until 24 DAS. Plants were UV-B treated 14 DAS (indicated with an arrow).

(D) to (F) Relative average leaf area (D), cell area (E), and estimated cell number (F) of fully expanded leaf #5 from UV-B-treated versus control Col-0 and *rGRF3* *Arabidopsis* plants. Results represent the average of 10 biological replicates \pm SE. Different letters indicate statistically significant differences applying the Student's *t* test ($P < 0.05$).

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experimental conditions, *uvr8* mutants show a similar relative decrease in leaf #5 area and estimated cell number in response to UV-B radiation as wild-type plants, while the palisade cell area is not affected, which was also observed for wild-type plants (Figure 7A). Moreover, *uvr8* shows similar levels of miRNA396 to that in the wild type in actively dividing leaves; besides, it is

similarly induced after the UV-B radiation treatment (Figure 7D). Thus, cell division inhibition in proliferating leaves by UV-B radiation is independent of UVR8, while other growth effects already reported to be produced by this radiation in plants, for example, the inhibition of cell expansion, may be dependent on this photoreceptor (Figure 8).

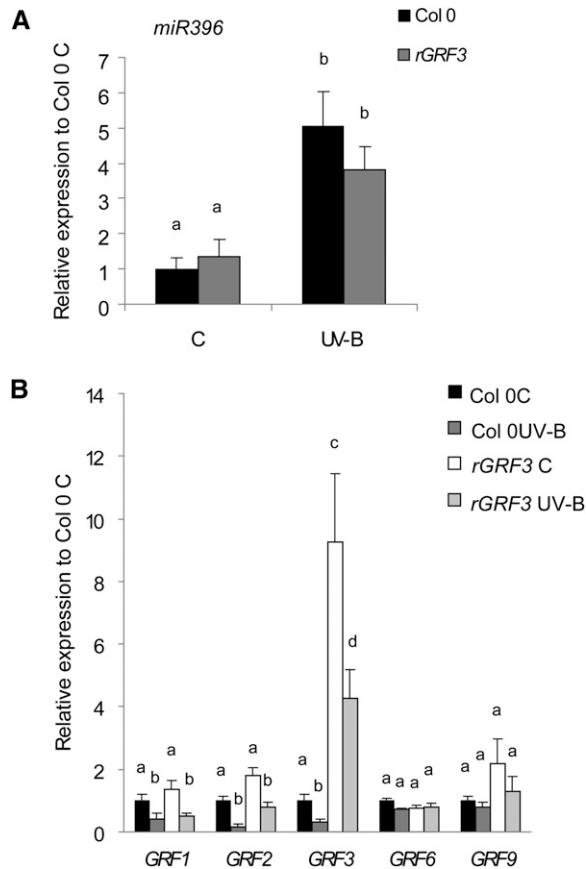


Figure 6. UV-B Effect on Expression Levels of *GRFs* and miR396 in Leaf #5 of Col-0 and *rGRF3* Plants.

(A) Expression levels relative to Col-0 control of miR396 in leaf #5 of Col-0 and *rGRF3* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} determined by qRT-PCR.

(B) Expression levels relative to Col-0 control of *GRF1*, *GRF2*, *GRF3*, *GRF6*, and *GRF9* genes determined by qRT-PCR analysis in Col-0 and *rGRF3* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} (UV-B). Results represent the average of six biological replicates \pm se. Different letters indicate statistically significant differences applying ANOVA test ($P < 0.05$).

On the other hand, the response to UV-B radiation as an environmental stress involves MPK3 and MPK6 signaling cascades (González Besteiro et al., 2011; González Besteiro and Ulm, 2013). In addition, ATR is a key regulator of the UV-B DNA damage response, and *Arabidopsis atr* mutants are hypersensitive to UV-B radiation (Culligan et al., 2004). Therefore, we investigated if miR396-regulated inhibition of cell proliferation by UV-B radiation is dependent on MPK3, MPK6, and/or ATR, using *mpk3*, *mpk6*, and *atr* knockout mutant plants (González Besteiro and Ulm, 2013). While *atr* and *mpk6* plants show a similar decrease in the rosette and leaf area as Col-0 plants in response to UV-B radiation (see Supplemental Figure 6 online; Figure 7), *mpk3* mutants that were UV-B irradiated for 4 h have

a bigger rosette area than Col-0 plants (see Supplemental Figure 6 online). Moreover, in *mpk3* UV-B-irradiated plants, the average leaf #5 area is larger than that of Col-0 plants, and this is because *mpk3* leaf #5 has more cells than Col-0 leaves, while the average cell area remains similar (Figure 7C). In addition, leaf #5 from *mpk3* plants has lower levels of miR396 and higher levels of *GRF1*, *GRF2*, and *GRF3* than those of Col-0 plants after the UV-B treatment (Figures 7D and 7E). Similarly as in *MIM396* plants (Figure 4A), although miR396 is still UV-B regulated in *mpk3* mutants, its levels remained significantly lower than those in Col-0 leaves (Figure 7D). Therefore, cell division inhibition in developing leaves with proliferating cells by UV-B radiation requires MPK3 (Figure 8) and is independent of the stress-activated MPK6 and ATR.

DISCUSSION

In this article, we present evidence that demonstrates that a single UV-B radiation treatment with irradiances of 2 Wm^{-2} during 4 h can affect *Arabidopsis* growth by inhibiting cell proliferation in developing organs and that this inhibition is, at least in part, regulated by miR396-mediated repression of *GRFs*. In Col-0 *Arabidopsis* plants, UV-B radiation induces the premature accumulation of miR396 in leaf #5, which contains actively proliferating cells at the moment of the treatment, and this increase parallels a decrease in the expression of *GRF1*, *GRF2*, and *GRF3*. Moreover, transgenic plants that express an artificial target mimic directed against miR396 and plants that express a miR396-resistant copy of *GRF2* or *GRF3* under their own promoter show a less sensitive phenotype to UV-B stress. In *MIM396* plants, *GRF1*, *GRF2*, and *GRF3* transcript levels are not decreased by UV-B radiation, as is observed in Col-0, demonstrating that the inhibition of cell proliferation observed in these plants requires the activity of this miR396. Moreover, plants with the *rGRF3* transgene do not show differences with Col-0 plants regarding the levels of miR396 or other *GRFs*, both under control conditions and after UV-B irradiation, showing that the effect of miR396 is through its action on *GRFs*. *rGRF2* plants show a similar phenotype to that of *rGRF3* plants after the UV-B treatment; therefore, the role of *GRFs* in cell proliferation during a UV-B treatment seems to be redundant, at least for *GRF2* and *GRF3*.

UV-B radiation has been shown to inhibit cell expansion in several species (Gonzalez et al., 1998; Ruhland and Day, 2000; Ruhland et al., 2005; Wargent et al., 2009a, 2009b). However, in our experiments, leaf growth inhibition in developing leaves with proliferating cells by UV-B radiation, mediated by miR396 and *GRFs*, is not due to the reduction of cell size; our treatment caused an inhibition of cell proliferation. The same UV-B treatment used in the experiments described in this article induces DNA damage in *Arabidopsis* plants; this damage is efficiently repaired a few hours after the end of the UV-B treatment (Lario et al., 2013; Campi et al., 2012). Therefore, to repair this damage, an arrest of the cell cycle may be necessary to prevent cells with damaged DNA from dividing, as was already proposed (Jiang et al., 2011b). In agreement with our results, changes in cell cycle markers and DNA damage transcripts under UV-B

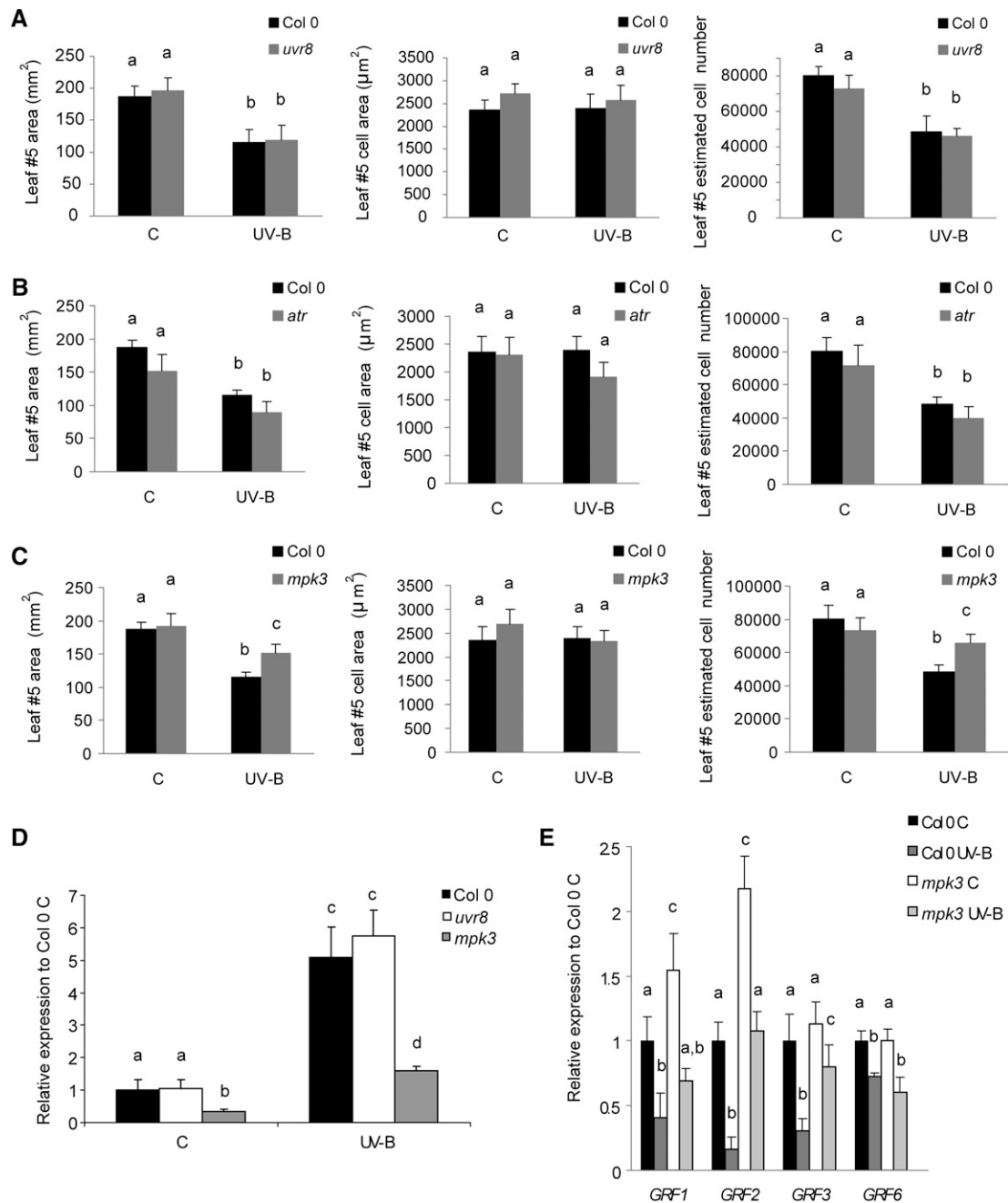


Figure 7. UV-B Effect in Cell Proliferation Is Independent of UVR8 and ATR but Dependent on MPK3.

(A) to (C) Col-0, *uvr8* (A), *atr* (B), and *mpk3* (C) mutant plants were treated with UV-B radiation for 4 h (2 Wm^{-2} ; right) or were kept under control conditions (C) in the absence of UV-B. Average leaf area, cell area, and estimated cell number of totally expanded leaf #5 from UV-B-treated versus control Col-0, *uvr8*, *atr*, and *mpk3* *Arabidopsis* plants.

(D) Expression levels relative to Col-0 control of miR396 in leaf #5 of Col-0, *uvr8*, and *mpk3* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} determined by qRT-PCR. Results represent the average of six biological replicates \pm SE. Different letters indicate statistically significant differences applying ANOVA test ($P < 0.05$).

(E) Expression levels relative to Col-0 control of *GRF1*, *GRF2*, *GRF3*, and *GRF6* genes determined by qRT-PCR analysis in Col-0 and *mpk3* plants under control conditions in the absence of UV-B (C) or immediately after a 4-h UV-B treatment at 2 Wm^{-2} (UV-B). Results represent the average of six biological replicates \pm SE. Different letters indicate statistically significant differences applying ANOVA test ($P < 0.05$).

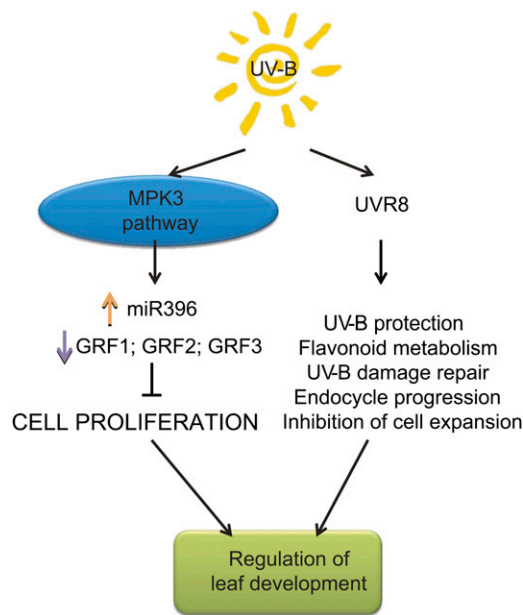


Figure 8. A Model Depicting the Effect of UV-B in the Regulation of Leaf Development.

The effect of UV-B radiation in cell proliferation inhibition is mediated by MPK3, miR396, and GRFs, but it is independent of the UV-B photoreceptor UVR8.

[See online article for color version of this figure.]

radiation were analyzed using actively dividing cells of *Arabidopsis* root tips (Jiang et al., 2011a). High levels of UV-B radiation induced expression changes of the cell cycle regulatory and DNA damage genes, suggesting that UV-B-induced DNA damage results in the delay of the G1-to-S transition of the cell cycle and that UV-B-induced G1-to-S arrest may be a protective mechanism that prevents cells with damaged DNA from dividing.

In our experiments, the inhibition of cell division by UV-B radiation is independent of the UV-B photoreceptor UVR8 and of ATR, a master regulator of UV-B-induced DNA damage, but requires the activity of MPK3. Two different pathways that participate in UV-B stress responses have been described in *Arabidopsis*. The first pathway is dependent on ATR, which is mainly active in the roots, and the second one is dependent on MPK3 and MPK6 and their phosphatase MKP1, with a primary role in leaves (González Besteiro and Ulm, 2013). In *Arabidopsis*, both MPK3 and MPK6 are activated by UV-B-induced DNA damage. Although there are several reports in which MPK3 and MPK6 were described to participate in the same stress response pathway (González Besteiro et al., 2011; González Besteiro and Ulm, 2013; Meng et al., 2013), in some studies, these kinases have been demonstrated to play different roles (Lumbreras et al., 2010; Kohorn et al., 2012). Interestingly, only MPK3 participates in UV-B inhibition of cell proliferation in our experiments, while *mpk6* mutants respond similarly to wild-type plants to our UV-B treatment. MKP1 has been suggested to have a function as a trigger of cell cycle checkpoints, and MKP1 positively regulates

CYCB1;1 expression in response to UV-B radiation in leaves (Cools and De Veylder, 2009; González Besteiro et al., 2011; González Besteiro and Ulm, 2013). Moreover, González Besteiro and Ulm (2013) demonstrated that pyrimidine dimers are a trigger of MAPK activation in *Arabidopsis*, as UV-B-mediated MPK3 and MPK6 activation is higher in photolyase mutants and in dark conditions that do not allow photorepair. Thus, pyrimidine dimers may play an important role as a signal of UV-B stress in the MPK3 activation pathway, therefore regulating miR396 and *GRF* expression to inhibit cell proliferation under these conditions.

In plants, regulation of cell proliferation is critical for leaf morphogenesis. Several reports have demonstrated that miR396-targeted *GRFs* are required for cell proliferation, and miR396 overexpression markedly decreases cell proliferation and the expression of cell cycle-related genes, indicating that miR396 negatively regulates cell proliferation by controlling entry into the mitotic cell cycle (Liu et al., 2009; Rodriguez et al., 2010; L. Wang et al., 2011). In addition, miR396 is mainly expressed in differentiated leaf cells arrested for cell proliferation, coinciding with its role in cell cycle regulation, suggesting that cell proliferation activity is mediated by miR396-targeted *GRFs* in *Arabidopsis* (Rodriguez et al., 2010). In agreement with our data, it is interesting to note that transgenic *Arabidopsis* plants overexpressing miR396 (with levels lower than fivefold increases) show decreased leaf area due to a decrease in the estimated cell number without changes in cell size when compared with Col-0 plants (Rodriguez et al., 2010).

On the other hand, Wargent et al. (2009b) previously characterized UVR8-dependent and independent responses on leaf morphogenesis at supplementary UV-B doses. For example, the inhibition of epidermal cell proliferation in response to UV-B radiation was UVR8 independent, while UVR8 is required for the regulation of endopolyploidy in response to UV-B radiation (Figure 8). In our experiments, UV-B effect on cell proliferation is independent on UVR8 (Figure 8), in agreement with the published data.

Several miRNAs have been involved in various stress responses, such as dehydration, mineral-nutrient, and even mechanical stress (reviewed in Lu and Huang, 2008). In particular, miR396 has been previously reported to be regulated by various stresses in different plants species, such as drought (Zhou et al., 2010; Kantar et al., 2011; Li et al., 2011; T. Wang et al., 2011), cold (Zhou et al., 2008), aluminium (Al^{3+} ; Chen et al., 2012), cadmium (Cd^{2+} ; Ding et al., 2011), salt, and alkali stress (Ding et al., 2009; Gao et al., 2010). For example, transgenic rice (*Oryza sativa*) and *Arabidopsis* plants constitutively overexpressing rice miR396c show reduced salt and alkali stress tolerance compared with that of wild-type plants (Gao et al., 2010). In addition, miR396 is strongly downregulated in cells giving rise to the syncytium in *Arabidopsis*, a plant root organ whose differentiation is induced by plant-parasitic cyst nematodes to create a source of nourishment (Hewezi et al., 2012). However, analysis of UV-B-regulated miRNAs from other studies have identified several miRNAs whose levels are changed by this radiation in *Arabidopsis* and *Populus tremula*, but miR396 was not identified in any of these analyses (Zhou et al., 2007; Jia et al., 2009). These results also demonstrate that UV-B effects on growth vary depending on the treatment, and it is possible

that miR396 can only be regulated by this radiation at intensities that provoke DNA damage and, therefore, cell cycle arrest.

In conclusion, here, we show that, at UV-B intensities that can induce DNA damage in *Arabidopsis* plants, leaf growth inhibition in proliferating tissues is a consequence of an inhibition of cell proliferation and is not because of an effect of this radiation on cell expansion. In addition, this inhibition is dependent on MPK3 and is mediated by miR396 and GRFs.

METHODS

Plant Material, Growth Conditions, and Irradiation Protocols

Arabidopsis thaliana ecotype Col-0 was used for all experiments. Plants were grown in soil under long photoperiods in a growth chamber (16 h light/8 h dark) at 23°C. The miRNA target motif in *GRF3* and *GRF2* was altered, introducing synonymous mutations in a cloned wild-type genomic fragment using the QuikChange site-directed mutagenesis kit (Stratagene) as described by Rodriguez et al. (2010). Artificial *MIM396* was generated by gene synthesis (Mr. Gene; Debernardi et al., 2012). The *uvr8*, *mpk6*, *mpk3*, and *atr* seeds were a gift from Roman Ulm (University of Geneva, Switzerland). *rGRF2* plants were previously described by Rodriguez et al. (2010).

Plants were exposed for 4 h with UV-B radiation from UV-B bulbs on a single day (2 Wm^{-2} UV-B as well as 0.65 Wm^{-2} UV-A; Bio-Rad) in a growth chamber; control plants were treated with the same lamps covered with a polyester film. Alternatively, plants were exposed 2 h per day with UV-B radiation (2 Wm^{-2} UV-B and 0.65 Wm^{-2} UV-A) for 4 d, and samples were collected from UV-B-treated and control plants after 1 and 4 d of UV-B exposure. Leaves for qRT-PCR were collected immediately after the treatment, and leaves for phenotypic analysis of cell proliferation and cell expansion were collected at the end of leaf #5 expansion.

qRT-PCR

Total RNA was isolated from ~20 mg of tissue using the TRIzol reagent (Invitrogen) as described in the manufacturer's protocol. Then, 0.5 to 1.0 μg of total RNA was incubated with RNase-free DNase I (1 unit/mL) following the protocol provided by the manufacturer to remove possible genomic DNA. The RNA was reverse-transcribed into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen); oligo(dT) and stem-loop oligo for miRNA396 were used as primers. The resultant cDNA was used as a template for quantitative PCR amplification in a MiniOPTICON2 apparatus (Bio-Rad), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter and Platinum Taq polymerase (Invitrogen). Primers for each of the genes under study were designed using PRIMER3 software (Rozen and Skaletsky, 2000) in order to amplify unique 50- to 250-bp products (see Supplemental Table 1 online). Amplification conditions were performed under the following conditions: 2 min of denaturation at 94°C; 40 cycles at 94°C for 15 s, 58°C or 60°C for 30 s, and 72°C for 45 s, followed by a 10-min extension at 72°C. Three biological replicates were performed for each sample. Melting curves for each PCR were determined by measuring the decrease of fluorescence with increasing temperature (from 65 to 98°C). PCR products were run on a 2% (w/v) agarose gel to confirm the size of the amplification products and to verify the presence of a unique PCR product. Gene expression levels were normalized to that of *Arabidopsis* calcium-dependent protein kinase3 (see Supplemental Table 1 online). The expression of this gene has been previously reported to remain unchanged by UV-B radiation (Ulm et al., 2004).

Small RNA Analysis

RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was resolved on 17% polyacrylamide gels under denaturing conditions

(7 M urea). Blots were hybridized using radioactively labeled nucleic acid oligonucleotide probes designed against miR396 (Exiqon). Alternatively, miR396 levels were determined by stem-loop qRT-PCR, as described previously (Chen et al., 2005). The sequences of the oligonucleotides are listed in Supplemental Table 1 online.

Microscopy Observations

Leaves were fixed with formalin-acetic-alcohol and cleared with chloral hydrate solution (200 g of chloral hydrate, 20 g of glycerol, and 50 mL of deionized water) as described (Horiguchi et al., 2005), and silhouettes of leaf images were acquired through a differential interference contrast microscope as shown in Figure 3B. Leaf area was quantified using ImagePro image analysis software. Palisade leaf cells were observed using differential interference contrast microscopy, the density of palisade cells per unit area of this region was determined, and the area of the leaf blade was divided by this value to calculate the total number of palisade cells in the subepidermal layer. To determine the cell area, 20 palisade cells were measured in each leaf. Experiments were performed in duplicate with at least 10 leaves, obtaining similar results.

Rosette Area Quantification

Approximately 20 seeds per tray were sown, leaving enough space in between them to avoid superposition during plant growth. At 14 DAS, a group of plants were subjected to 2 or 4 h UV-B treatment (2 Wm^{-2}), and another group was kept as control plants; after the treatment, all the plants were kept in a growth chamber until the end of the experiment. Every 3 d, photographs were taken and total leaf area (rosette area) of each plant was measured using the ImageJ software.

GUS Assay

To visualize the activity of the reporter, the transgenic plants (*CYCB1;1:GUS*) were subjected to GUS staining as described by Donnelly et al. (1999).

Statistical Analysis

Data presented were analyzed using two-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Bonferroni, Holm-Sidak, Dunnett, and Duncan tests ($\alpha = 0.05$) using the Sigma Stat Package. When comparing two data sets, Student's *t* test was used ($n = 5$ to 10 biological replicates in a single experiment; $P < 0.05$), and significant differences are indicated with asterisks in Figures 1 and 2.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: GRF1, AT2G22840; GRF2, AT4G37740; GRF3, AT2G36400; GRF6, AT2G06200; GRF9, AT2G45480; miR396A, AT2G10606; miR396B, AT5G35407; UVR8, AT5G63860; ATR, AT5G40820; MPK3, AT3G45640; and MPK6, AT2G43790.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. *CYCB1;1:GUS* Reporter Expression in Plants at the Moment of UV-B Treatments.

Supplemental Figure 2. UV-B Effect on Expression Levels of miR396 in Different Tissues and at Different Developmental Times.

Supplemental Figure 3. Kinetics of miR396 and *GRF* Expression during Prolonged UV-B Treatment.

Supplemental Figure 4. Average Leaf Area, Cell Area, and Estimated Cell Number of Fully Expanded Leaf #5 from UV-B-Treated versus Control Col-0 and *rGRF3 Arabidopsis* Plants.

Supplemental Figure 5. UV-B Repression of Cell Proliferation Is Mediated by miR396.

Supplemental Figure 6. UV-B Effect on Cell Proliferation Is Independent of ATR and MPK6 but Dependent on MPK3.

Supplemental Table 1. List of Oligonucleotides Used in This Study.

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AUTHOR CONTRIBUTIONS

R.C., R.E.R., J.F.P., and P.C. designed the experiments and analyzed the data. R.C. did the UV-B experiments, qRT-PCR, and plant analysis. P.C. wrote the article. R.E.R., J.M.D., and J.F.P. constructed the transgenic plants and designed oligonucleotides for miR396 and GRF measurements.

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