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Flavonols protect Arabidopsis plants against UV-B deleterious effects

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Running title: Flavonols in UV-B protection

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Dear Editor,

Flavonols are synthesized by flavonol synthase (FLS) enzymes (Martens et al., 2010). These compounds absorb UV-B light in the 280–320 nm region, and their concentration increases in plants exposed to environmental abiotic and biotic stresses, including UV-B; consequently, flavonols are thought to act as UV-B filters (Agati et al., 2011). It has been also suggested that these metabolites function as reactive oxygen species (ROS) scavengers, as they contain an OH- group in the 3-position of the flavonoid skeleton, which allows them to chelate metals, inhibiting the formation of free radicals and ROS accumulation, once formed (Agati et al., 2009). For these reasons, it has been suggested that flavonols play uncharacterized roles in UV responses (Verdan et al., 2011). Nevertheless, despite the fact that the role of flavonols in UV-B protection has been inferred; the protection conferred by flavonols on the target sites of UV-B-damage has not been directly proven *in planta*.

Recently, we demonstrated that maize FLS1 (*Zm*FLS1) complements the flavonol deficiency of the Arabidopsis *fls1* mutant and decreases its high level of anthocyanins, characteristic of this mutant plant (Falcone Ferreyra et al., 2010). In order to demonstrate that flavonols protect plants against UV-B damage, we generated Arabidopsis transgenic plants overexpressing the maize *FLS1* cDNA (*35S:ZmFLS1*), and evaluated different responses of these transgenic plants against UV-B damage.

Transformed plants were selected by hygromycin-resistance, and the presence of the transgene was verified by PCR analysis on genomic DNA, showing independent transgenic lines different mRNA levels of the transgene by RT-PCR and RT-qPCR analysis (Supplemental Figure 1). Flavonol levels in *35S:ZmFLS1* and Col 0 plants were analyzed by HPLC; Figure 1A shows that transgenic plants exhibit significantly higher levels of both kaempferol (K) and quercetin (Q) than WT plants. However, whereas kaempferol levels increased 3.8 times, only a 1.7-fold increase was measured for quercetin. This result is in agreement with our previous data that shows that ZmFLS1 has 4 times higher affinity for dihydrokaempferol (k_{cat}/K_m= 111.8 μm^{-1} sec^{-1}) than for dihydroquercetin (k_{cat}/K_m= 27.4 μm^{-1} sec^{-1}, Falcone Ferreyra et al., 2010). On the other hand, this difference in K and Q accumulation in the transgenic plants could be due to the flavonoid 3' hydroxylase (F3'H) activity, which could be responsible to limit the availability of DHQ substrate for ZmFLS1 (Supplemental Figure 1E). Surprisingly; transgenic and WT plants exhibited similar levels of

anthocyanins (Supplemental Figure 2A). This data differs from previous results using transgenic Arabidopsis plants overexpressing the endogenous FLS1, which showed decreased levels of anthocyanins (Kuhn et al., 2011). Moreover, the overexpression of *AtFLS1* in Arabidopsis did not result in an increase in the flavonol content as we observed in our transgenic plants, reflecting significant kinetic differences between FLS1 enzymes from Arabidopsis and maize. However, we cannot rule out that our transgenic plants may have altered levels of other metabolites.

Then, the effect of increased flavonol levels protecting DNA against damage after a 4h-UV-B treatment was analyzed. Figure 1B shows cyclobutane pyrimidine dimers (CPD) accumulation by an immunological assay in samples from WT and *35S:ZmFLS1* plants after the UV-B treatment. In the absence of UV-B, CPD levels in WT and the transgenic plants were similar. After 4h of exposure with UV-B, unrepaired lesions accumulated in all plants; however, the accumulation of CPDs in WT plants was more severe than in plants with higher flavonol levels, demonstrating that flavonols protect the plants against DNA damage induced by UV-B. Furthermore, the major CPD removal mechanisms were unaffected in the transgenic plants, showing similar levels of transcripts of some DNA repair enzymes as WT plants (Supplemental Figure 2). Moreover, Arabidopsis transgenic plants expressing *ZmFLS1* fused to the green fluorescent protein (*35S:ZmFLS1-GFP*) showed localization of FLS1-GFP, not only in the cytosol, but also in the nuclei (Figure 1C), as already described for *At*FLS1-GFP in Arabidopsis under its own promoter (Kuhn et al., 2011), suggesting that flavonols may accumulate in this organelle. In this way, flavonols may directly protect DNA against UV-B induced damage in the nuclei. Thus, increased levels of flavonols protect the transgenic plants against DNA damage by UV-B, probably through a direct protection of flavonols in the nucleus.

To investigate the effect of increased flavonol levels against UV-B damage to the photosynthetic machinery, the maximum efficiency of photosystem II (PSII, Fv/Fm), quantum yield of PSII (ϕ_{PSII}), CO_2 fixation and chlorophyll levels were assessed immediately after the UV-B treatment. In control conditions without UV-B, all photosynthetic parameters were similar regardless of flavonol levels (Figure 1D, E). After the UV-B treatment, both the WT and the transgenic plants showed a decrease in the maximum efficiency of PSII and the ϕ_{PSII} (Figure 1D, E); however, this decrease is significantly more pronounced in the WT than in the transgenic plants. In addition, while the WT plants showed a significant decrease in both chlorophyll A and B after

the treatment, UV-B did not affect chlorophyll levels in the transgenic plants (Figure 1F and G). Therefore, flavonols also protect the plants against damage of the photosynthetic electron transport machinery. Nevertheless, CO_2 fixation followed a small but significant decline in photosynthesis rate after UV-B irradiation in the WT and the transgenic plants, both under 100 and 250 µmol m^{-2} s^{-1} (Supplemental Figure 2), suggesting that the protective role of flavonols is not effective for this metabolic process, at least in the experimental conditions tested. Therefore, both photosynthetic processes seem to be affected differently by UV-B, in our experiments flavonols proved to be more important protecting against PS II damage than in CO_2 fixation.

Polyunsaturated fatty acids, the main components of biological membranes, are highly susceptible to peroxidation by ROS, finally resulting into the formation of highly toxic aldehydes that react with thiobarbituric acid generating TBARS. Thus, damage to lipids was assessed by measuring the TBARS content in WT and transgenic plants expressing *ZmFLS1.* After the 4h-UV-B treatment, *35S:ZmFLS1* plants showed a lower increase in lipid peroxidation levels than WT plants (Figure 1H), while under control conditions, basal levels of TBARS were similar among both plants. UV-B sensitivity was also analyzed by electrolyte leakage and inhibition of primary root elongation assays (Tong et al., 2008). WT seedlings showed a significant increase in leaf electrolyte leakage; while *35S:ZmFLS1* plants showed only a minor effect by UV-B (Figure 1I). These results are probably due to an increased oxidative stress induced by UV-B, resulting in the production of ROS. Consistently, ROS production is usually correlated with the inhibition of photosynthesis, which was measured in our experiments (Figure 1). Furthermore, our results support the hypothesis postulated in which flavonols could be substrates of vacuolar peroxidases, scavenging H_2O_2 that diffuses from the chloroplast, acting as a sink/buffer of H_2O_2 levels in plant cells (Ferreres et al., 2011).

Finally, while WT plants showed a significant decrease in primary root elongation after the UV-B treatment that was evident 2 days after the end of the treatment (Figure 1J); plants with increased flavonol levels displayed a significant lower decrease in primary root growth than WT plants, which was observed later than for WT plants (3 days after the end of the UV-B treatment). It is interesting to note that the transgenic seedlings had a shorter primary root than WT seedlings under control conditions in the absence of UV-B (Figure 1J and Supplemental Figure 3D); despite this, after the UV-B treatment both WT and transgenic roots have a similar length,

demonstrating that UV-B affects root elongation rate more significantly in WT than in transgenic seedlings.

Flavonols have also been involved in the regulation of auxin transport (Kuhn et al., 2011). Thus, we also analyzed if alterations in flavonol levels affected auxin-related developmental processes. Arabidopsis plants expressing *ZmFLS1* show an overall increase in the rosette size and have bigger leaves than WT plants, which is a consequence of plants having bigger cells; while the number of cells per leaf is similar to WT plants (Supplemental Figure 3A-C). On the contrary, the transgenic plants have a shorter primary root than WT plants (Supplemental Figure 3D). Although more experiments need to be done to demonstrate that our transgenic plants have altered auxin transport, the developmental phenotypes exhibited are consistent with an altered auxin distribution.

Overall, the results presented here indicate that Arabidopsis transgenic plants expressing *ZmFLS1* are less sensitive to UV-B radiation than WT plants. Thus, we demonstrate that flavonols are effective UV-B sunscreens. Induction of the synthesis of these compounds can protect plants against this stress, and this role could be achieved not only by their UV-absorbing characteristics, but also reducing ROS once formed.

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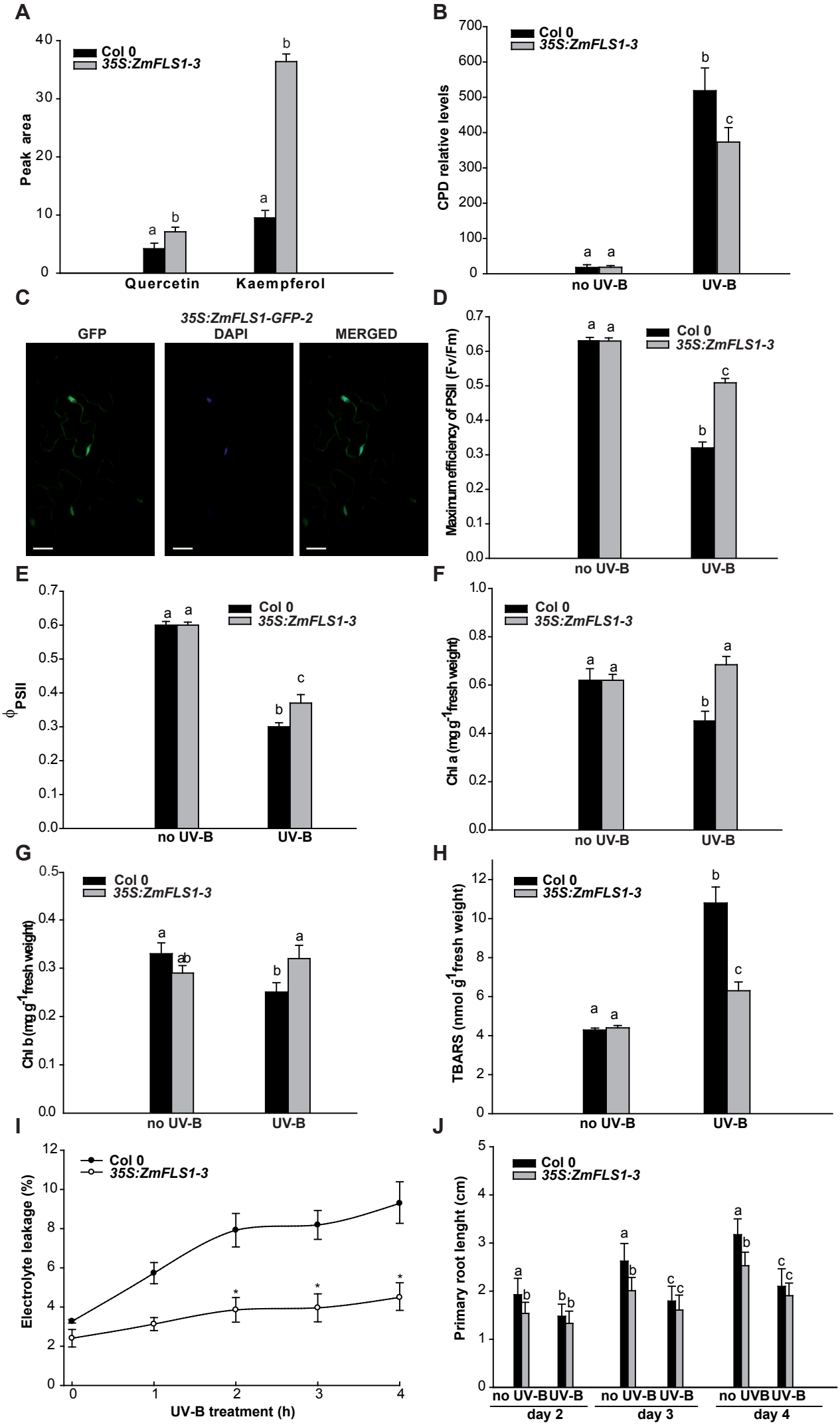
A
Col 0
35S:ZmFLS1-3
Peak area
Quercetin
Kaempferol
B
Col 0
35S:ZmFLS1-3
CPD relative levels
no UV-B
UV-B
C
35S:ZmFLS1-GFP-2
GFP
DAPI
MERGED
D
Col 0
35S:ZmFLS1-3
Maximum efficiency of PSII (Fv/Fm)
no UV-B
UV-B
E
Col 0
35S:ZmFLS1-3
ϕPSII
no UV-B
UV-B
F
Col 0
35S:ZmFLS1-3
Chl a (mg g-1fresh weight)
no UV-B
UV-B
G
Col 0
35S:ZmFLS1-3
Chl b (mg g-1fresh weight)
no UV-B
UV-B
H
Col 0
35S:ZmFLS1-3
TBARS (nmol g-1fresh weight)
no UV-B
UV-B
I
Col 0
35S:ZmFLS1-3
Electrolyte leakage (%)
UV-B treatment (h)
J
Col 0
35S:ZmFLS1-3
Primary root lenght (cm)
no UV-B UV-B
day 2
day 3
day 4

FIGURE LEGENDS:

Figure 1: Higher flavonol levels in *Arabidopsis thaliana* transgenic plants expressing *ZmFLS1* result in increased protection against UV-B damage. (A) Quantification of flavonol peaks in WT (Col 0) and *35S:ZmFLS1-3* transgenic plants. The quantification is based on peak area (μVol*sec) measurements of HPLC chromatograms revealed at 360 nm. For each flavonol analyzed, different letters indicate a significant difference at $P<0.05$. **(B)** CPD levels in DNA of WT and transgenic *35S:ZmFLS1-3* plants under control conditions without UV-B (no-UV-B) and after a UV-B treatment for 4h. Experiments were done under conditions that allowed photorepair in the light. 1.5 μg of DNA was loaded in each well. Results represent the average ± SEM of six independent biological replicates. Different letters denote statistical differences ($P<0.05$) applying ANOVA tests using Sigma Stat 3.1. **(C)** Subcellular localization of *35S:ZmFLS1-GFP-2* (T2.2 line). Confocal laser-scanning micrographs showing localization of *Zm*FLS1-GFP in Arabidopsis leaf epidermal cells. FLS1-GFP was most easily detected in the cytosol and the nuclei of cells. Green represents FLS1-GFP fluorescence, while blue represents DAPI fluorescence. Images were merged to show signal overlap. Scale bars represent 20 μm. Maximum efficiency of PSII **(D)**, quantum yield of PSII (ϕ_{PSII}) **(E)**, and chlorophyll A **(F)** and B **(G)** levels of WT and *35S:ZmFLS1-3* transgenic plants under control conditions without UV-B (no-UV-B) and after a UV-B treatment for 4h. Measurements are the average of six adult leaves from six different plants. Error bars represent SEM. Different letters denote statistical differences ($P<0.05$) applying ANOVA tests using Sigma Stat 3.1. TBARS levels **(G)**, electrolyte leakage **(H)** and inhibition of primary root elongation **(I)** of WT and *35S:ZmFLS1-3* transgenic plants under control conditions without UV-B (no-UV-B) and after a UV-B treatment. The experiments were done in triplicate using six different plants, and results represent the average ± SEM. Statistical significance was analyzed applying ANOVA analysis with $P < 0.05$ using Graphpad Prism 5.03 software. Significant differences from WT plants are marked with different letters **(G)** and asterisks **(H)**. **(I)** For each day after UV-B treatment, different letters indicate a significant difference from the control condition (no UV-B).