

Rapid transcriptome responses of maize (*Zea mays*) to UV-B in irradiated and shielded tissues

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Abstract

Background: Depletion of stratospheric ozone has raised terrestrial levels of ultraviolet-B radiation (UV-B), an environmental change linked to an increased risk of skin cancer and with potentially deleterious consequences for plants. To better understand the processes of UV-B acclimation that result in altered plant morphology and physiology, we investigated gene expression in different organs of maize at several UV-B fluence rates and exposure times.

Results: Microarray hybridization was used to assess UV-B responses in directly exposed maize organs and organs shielded by a plastic that absorbs UV-B. After 8 hours of high UV-B, the abundance of 347 transcripts was altered: 285 were increased significantly in at least one organ and 80 were downregulated. More transcript changes occurred in directly exposed than in shielded organs, and the levels of more transcripts were changed in adult compared to seedling tissues. The time course of transcript abundance changes indicated that the response kinetics to UV-B is very rapid, as some transcript levels were altered within 1 hour of exposure.

Conclusions: Most of the UV-B regulated genes are organ-specific. Because shielded tissues, including roots, immature ears, and leaves, displayed altered transcriptome profiles after exposure of the plant to UV-B, some signal(s) must be transmitted from irradiated to shielded tissues. These results indicate that there are integrated responses to UV-B radiation above normal levels. As the same total UV-B irradiation dose applied at three intensities elicited different transcript profiles, the transcriptome changes exhibit threshold effects rather than a reciprocal dose-effect response. Transcriptome profiling highlights possible signaling pathways and molecules for future research.

Background

The evolution of terrestrial life was possible after the formation of a stratospheric ozone layer that absorbed most of the ultraviolet-B (UV-B) radiation (280-315 nm) in sunlight [1]. Recent depletion of stratospheric ozone catalyzed by chlorofluorocarbons and other pollutants has raised terrestrial UV-B levels, an environmental change linked to increased risk of skin cancer [2]. This also has potentially deleterious

consequences for plants, including decreases in crop yields [3-5]. Because plants must be exposed to sunlight to power photosynthesis, they are inevitably exposed to the damaging UV-B. Adaptations include both protection, such as accumulation of UV-absorbing pigments [6-8], and damage repair, such as the use of UV-A photons to reverse some types of UV-induced DNA lesions [9]. Because of its absorption spectrum, DNA is a major and long-studied target of UV-B damage:

even low doses of radiation can kill plant mutants that lack specific DNA repair pathways [9,10]. UV-B can also directly damage proteins and lipids [11], and we recently found that UV-B radiation crosslinks RNA to particular ribosomal proteins, with a concomitant decrease in translation (P.C. and V.W., unpublished work).

In addition to damaging existing cellular constituents, UV-B induces the rapid activation of *c-fos* and *c-jun* in mammalian cells [12,13]. Induction is mediated through several cytoplasmic signal transduction pathways [14,15], including multiple MAP kinase pathways. After UV-B irradiation, plants display diverse morphological and physiological responses [3-5] that are likely to involve multiple signal transduction cascades. Changes in intracellular calcium, calmodulin, serine/threonine kinases, and phosphatase activities have been implicated in UV-B-mediated transcriptional activation of chalcone synthase, the first gene in the flavonoid sunscreen biosynthetic pathway [16,17]. In addition, UV-B has been proposed to act through the octadecanoid pathway in tomato to stimulate the expression of genes encoding antimicrobial defenses [18]. Recently, two highly homologous MAP kinases, LeMPK1 and LeMPK2, were found to be activated in response to different stresses, including UV-B radiation, in suspension cell cultures of the wild tomato, *Lycopersicon peruvianum*, while an additional MAP kinase, LeMPK3, was only activated by UV-B radiation [19]. Therefore, some UV-B signal pathways are shared with other environmental perturbations, while additional pathways may account for UV-B-specific responses. Despite these observations, the mechanism(s) by which UV triggers intracellular signaling pathways remains poorly understood in both mammalian and plant cells. Candidate triggering molecules include reactive oxygen species (ROS) such as singlet oxygen, superoxide radicals, hydroxyl radicals, and H₂O₂, all of which are increased in response to UV and may be key regulators of UV-induced signaling pathways [20-22]. One mechanism through which ROS can activate signal transduction in animal cells is ligand-independent activation of membrane receptors, which can result from oxidation of receptor-directed protein tyrosine phosphatases [23].

In initial analyses using microarrays containing approximately 2,500 maize cDNAs, we documented the physiological acclimation responses in adult maize leaves (*Zea mays*) grown without UV-B or UV-A+B in sunlight for 20 days and for 1 day after the UV solar spectrum was restored. In the leaves shielded from UV, 304 transcripts were identified that had altered abundance compared to control leaves exposed to the full spectrum of sunlight during the depletion regime or after 1 day of UV exposure [24]. A comparison among near-isogenic lines with varying levels of flavonoid sunscreen indicated that the *b, pl* anthocyanin-deficient line maize showed a greater response than anthocyanin-containing lines [24]. This is as expected if this anthocyanin pigment is a sunscreen that attenuates UV-B dosage [6]. Confirming previous studies on individual genes, several stress-related pathways were

shown to be upregulated by UV-B whereas genes encoding products required for photosynthesis were downregulated [24]; the latter result has also been obtained through transcriptome profiling in *Nicotiana longiflora* [25]. In addition, dozens of candidate genes and pathways were identified that had not been previously associated with acclimation to UV-B [26].

With the goal of understanding the integrative processes involved in UV-B acclimation that result in altered plant morphology and physiology, we investigated gene expression at several UV-B fluence rates and exposure times in multiple organs of maize. Given its heightened sensitivity to UV-B and its similarity to commercial maize varieties that have been bred to lack anthocyanin, the *b, pl* anthocyanin-deficient line was used. The B and Pl transcription factors strongly induce expression of chalcone synthase, the first enzyme in the flavonoid biosynthetic pathway, and subsequent steps leading to anthocyanin pigments [27]. After exposure to UV-B for as little as an hour, transcript changes are detectable in the *b, pl* genotype both in directly exposed leaves and in roots. These results indicate that there are systemic, integrated responses to supplemental UV-B. Transcriptome profiling also highlighted possible signaling pathways and molecules for future research.

Results

Microarray experimental design and hybridization reliability

To examine gene activity changes elicited by UV-B radiation in different maize organs, microarray hybridization experiments were used to determine steady-state mRNA levels using Unigene I arrays from the Maize Gene Discovery Project. The slides contained 5,664 maize cDNAs printed in triplicate spots (for more information see [28]); 90% of the elements showed hybridization above background with adult leaf cDNA probes. We examined patterns of gene expression in adult leaves, seedling leaves, emerging tassel, 14-day-old roots, and immature ears after whole plants were subjected to 8 hours exposure under UV-B lamps with a biologically effective UV irradiance of 0.36 W/m² (9 kJ/m²/day) normalized to 300 nm [29]. Transcript levels were analyzed in duplicate biological samples harvested immediately after the UV-B treatment and in control plants treated identically except for UV-B supplementation. UV-B-treated and control cDNA samples were differentially labeled with Cy3 and Cy5 and compared by microarray hybridizations in duplicate dye-swapping experiments, which also provided a further repetition of each comparison. Reproducibility between hybridizations was excellent, with the correlation coefficients of the ratios greater than 0.95 in all cases (Figure 1). The mean hybridization signal strength and the standard error of the mean were calculated as an average of the signal intensity of each triplicate spot within the same and duplicate hybridizations. Thus, for each expressed sequence tag (EST) queried,

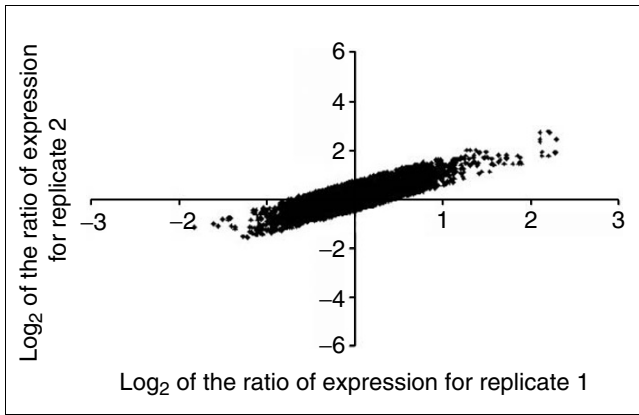


Figure 1
Microarray analysis of gene expression changes after UV-B exposure. Scatter plot comparing ratios of signal values from two replicate microarray hybridizations with Cy3-dUTP-labeled and Cy5-dUTP-labeled mRNA from adult leaves of *b*, *pl* plants after 8 h exposure under UV-B lamps and under no UV-B. Data from images of dye-swapping experiments were plotted as the mean intensity after normalization of ESTs spotted in triplicate.

we analyzed transcript levels in six independent spots. During the analysis, only changes in mRNA abundance in excess of twofold of controls in all replicate experiments were accepted as significant.

UV-B supplementation effects on gene expression in individual maize organs

Using these criteria, 347 ESTs were identified that showed significant differential expression in response to UV-B treatment in at least one organ after plants were irradiated for 8 hours; this corresponds to 6% of the total probe set (Figure 2). Of these, 285 were upregulated by UV-B, while 80 were scored as downregulated. It is important to note that the total number of UV-B-regulated genes is lower than the sum of up- and downregulated genes, because 18 ESTs that increased in some organs were downregulated by UV-B in others.

As summarized in Figure 2, the greatest overall response was observed in adult tissues: emerging tassels (162 transcripts up, 4 down) and mature leaves (121 up, 16 down). In contrast, seedling leaves (62 up, 17 down) showed fewer significant changes than adult leaves. Directly exposed organs had many more transcripts with significant increases in expression relative to the non-UV-B irradiated control than transcripts with lower expression. Shielded organs experienced little or no direct UV-B, but nonetheless exhibited transcriptome alterations. Roots in soil showed increases in 9 and decreases in 25 transcripts (Figure 2). Some transcripts downregulated in roots were upregulated by UV-B in tissues directly exposed to radiation (see Additional data file 1). Immature ears before silk emergence are shielded by multiple layers of husk leaves; nevertheless, 34 genes were downregulated by UV-B, while 8

were upregulated. Because roots and ears receive little or no direct radiation, organs directly exposed to UV-B probably produce signals that are transmitted to shielded organs, where they elicit distinct transcriptome changes, primarily decreases in transcript abundance.

Figure 3 shows that there is little overlap between UV-B-regulated transcripts in the five sample types. In the directly irradiated organs, 26 ESTs were upregulated in both seedling and adult leaves, and 36 showed increased levels in both emerging tassels and adult leaves. Only six transcripts (an omega-6 fatty acid desaturase, GenBank accession number AW065914; a cytochrome *b5*, AW144935; a glutamine synthetase, AI947856; two ribosomal proteins, L11, AI948309 and P0, AW231530; and a putative protein, AI861109; see Additional data file 1) showed upregulation in all three irradiated tissues. Similarly, in the two shielded organs only eight transcripts were downregulated in both ear and root.

Patterns of expression changes after UV-B supplementation in different tissues

Genes were grouped according to similarity of expression patterns by two algorithms: self-organizing maps (SOMs) (Figure 4a), and hierarchical clusters incorporating both patterns and expression amplitudes (Figure 4b). We found that genes assigned to key SOM clusters (Figure 4a) are also close in the hierarchical clustergram (Figure 4b), indicating that the independent methods yield consistent depictions. Several SOM clusters were analyzed in detail. First, SOM co includes transcripts that are downregulated by UV-B in adult leaves.

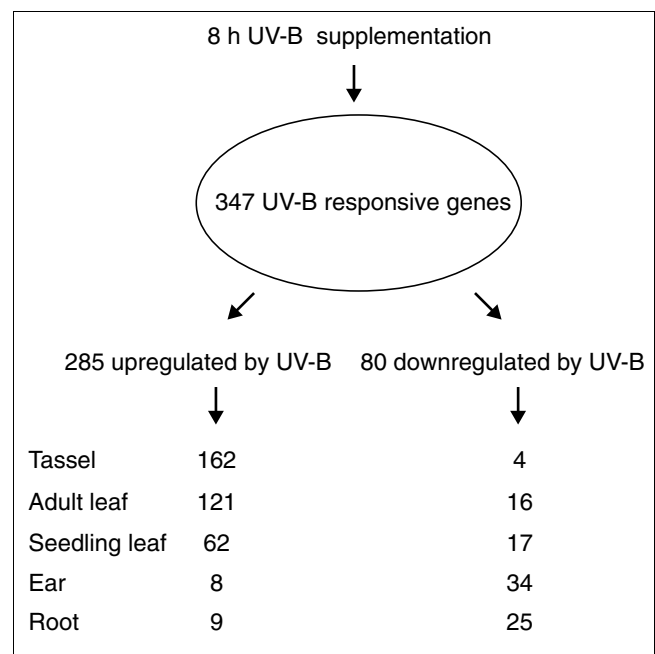
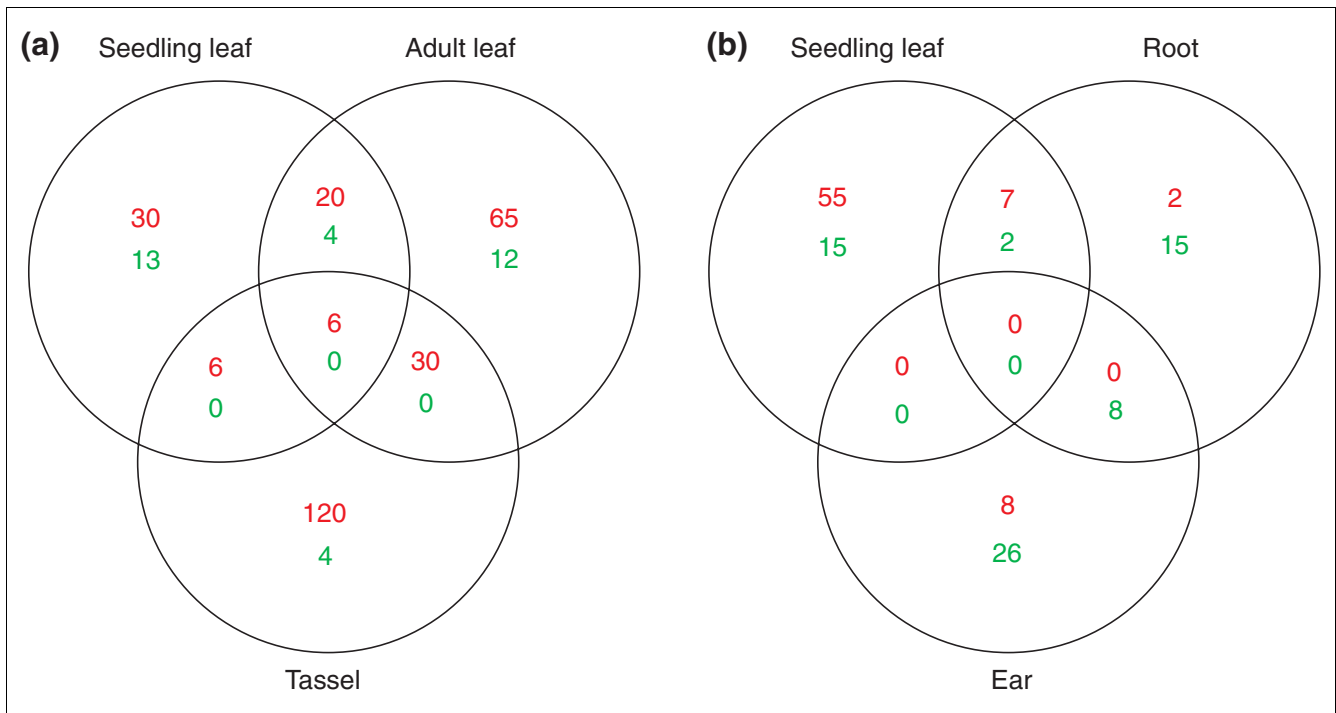


Figure 2
Summary of the number of ESTs responsive to UV-B supplementation in different tissues of *b*, *pl* maize plants.

**Figure 3**

Venn diagrams of comparisons between UV-B-responsive genes in different tissues of maize. Upregulated genes are colored red, downregulated genes are colored green. Sets of genes were selected using the criteria described in Materials and methods. **(a)** Intersection of genes regulated by UV-B in UV-B-exposed tissues (seedling and adult leaves and emerging tassels). **(b)** Intersection of genes regulated by UV-B in UV-B shielded tissues (roots and immature ears) and seedling leaves.

Transcripts for RuBisCO small subunit, a photosystem II 22 kDa polypeptide, and a photosystem I P700 apoprotein A2 are in this cluster (Figure 4a; see Additional data file 1 for complete listings of genes responding to each SOM cluster). Transcripts encoding proteins related to photosynthesis and CO₂ fixation, such as RuBisCO, and proteins of both photosystems I and II were previously shown to decrease after UV-B radiation in adult leaves [24]; downregulation of photosynthetic proteins has also been documented in pea and wheat [30,31] and in *Nicotiana longiflora* [25]. Surprisingly, these transcripts were unaffected in seedling leaves, an illustration of the greater sensitivity to UV-B radiation of adult compared to seedling leaves.

SOM c4 includes eight ribosomal protein genes upregulated by direct exposure to UV-B in adult tissues - both leaves and

tassels (Figure 4a; and see Additional data file 1). In previous studies, we found that the functional group with the largest number of genes upregulated by UV-B was that encoding proteins involved in translation [24]. Because RNA strongly absorbs UV photons, *in vitro* irradiation causes formation of crosslinks in ribosomal RNA and between mRNA, tRNA, rRNA and proteins [32]. We determined that UV-B radiation crosslinks RNA and four specific ribosomal proteins *in vivo*; concomitantly, overall translation is decreased by UV-B, suggesting that ribosome damage *in vivo* occurs after UV-B exposure (P.C. and V.W., unpublished work). As a consequence, coordinated upregulation of ribosomal protein synthesis is likely to be important for the restoration of this crucial cellular function by *de novo* ribosome synthesis. The novel discovery here is that this upregulation occurs not only in adult leaves but also in tassels; however, neither seedling leaves nor

Figure 4 (see following page)

Analysis of microarray data. Self-organizing map (SOM) clusters of expression profiles **(a)** and cluster analysis of transcripts **(b)** from maize tissues showing different UV-B responses. RNA from the same tissues not exposed to UV-B was used as the reference. **(a)** Each graph displays the mean pattern of expression of the ESTs in the cluster in blue and the standard deviation of average expression (red and yellow lines). The number of ESTs in each cluster is at the bottom left corner of each SOM. The y-axis represents log₂ of gene-expression levels. **(b)** Clustering was performed according to [43]. The color saturation reflects the magnitude of the log₂ expression ratio (Cy5/Cy3) for each transcript. Red color means higher transcript levels than the reference, whereas green means lower transcript levels than the reference. Gray corresponds to flagged ESTs that had signals similar to the background in some conditions and hence were eliminated during the analysis. The color log₂ scale is provided at the bottom of the figure. Correspondence between nodes of the cluster tree and SOM clusters are indicated on vertical bars on the left side of the tree.

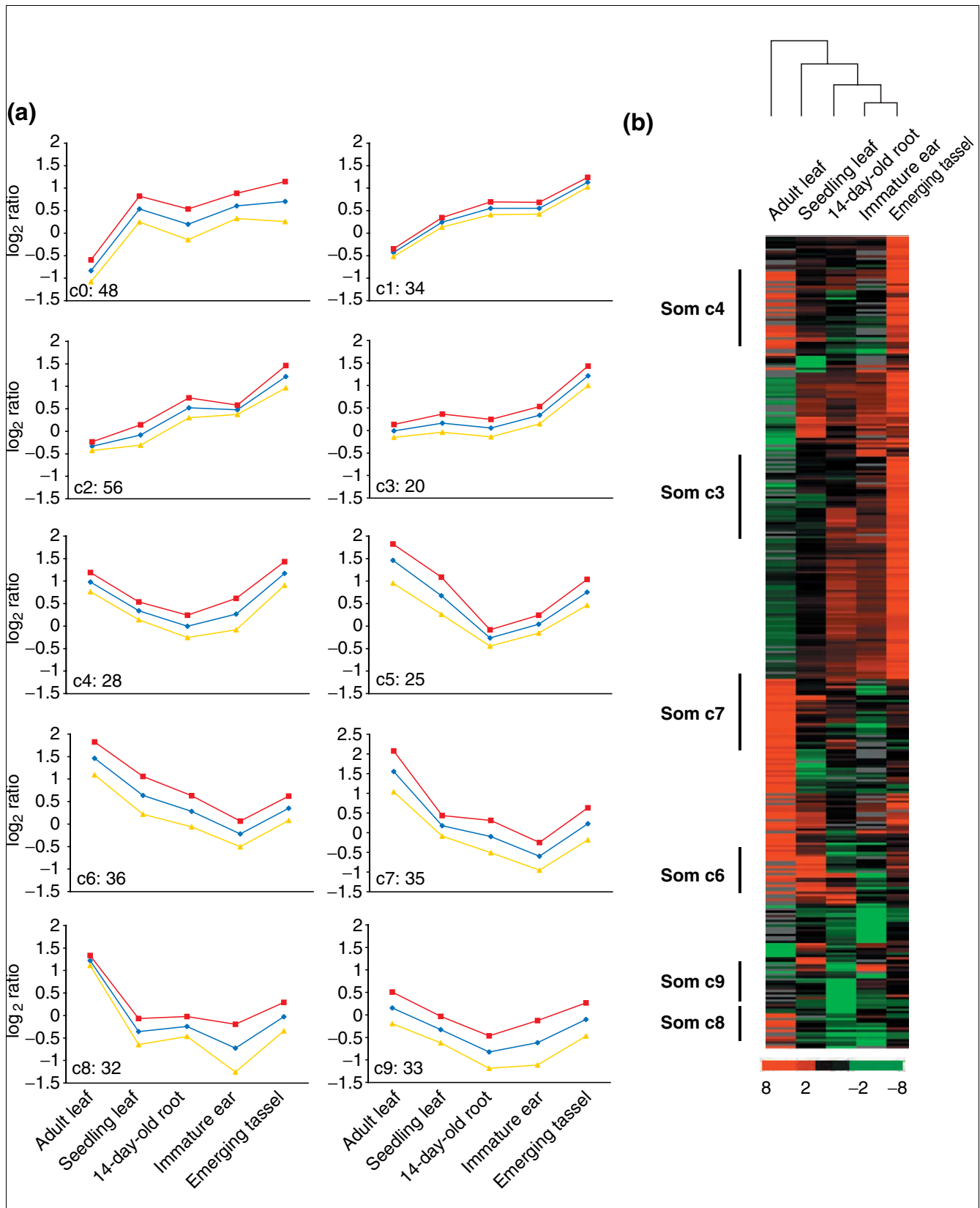


Figure 4 (see legend on previous page)

shielded tissues exhibit upregulation of ribosomal protein genes. Because seedling leaves lack both the downregulation of photosynthetic genes and upregulation of ribosomal protein genes characteristic of adult leaves, it seems that they are less affected by UV-B radiation.

SOM c6 includes 36 ESTs that are upregulated by UV-B in all leaves (Figure 4; and see Additional data file 2), and the identified genes correspond to three key processes: quality control of nucleic acids; protein turnover; and production of ROS. One example in the first category is a transcript with high homology to *Arabidopsis RAD17*. Genotoxic stress in yeast and human cells activates checkpoints that delay cell-cycle progression to allow DNA repair [33]. RAD proteins, including RAD17, are key to the early response during the activation of both DNA-damage repair and replication checkpoints. A similar role for this protein could be required in maize leaves after UV-B exposure. Other members of SOM c6 are important in the quality control of RNA; transcripts with homology to proteins involved in RNA maturation, such as Sm protein F and XRN2, are upregulated by UV-B.

UV-B causes crosslinking and oxidative damage to proteins [11], and a range of protein-turnover pathways are implicated in the UV-B response in maize. mRNAs for two proteinases are included in SOM c6 (a cysteine proteinase and a zinc-dependent protease). We previously found significant increases in the transcript levels of ubiquitin, ubiquitin-binding proteins, proteasome proteins and proteinases, together with several chaperonins, after UV-B exposure in maize as a function that is inversely correlated with flavonoid sunscreen content [24]. Considering these transcriptome profiling experiments together with the current results, an enhanced capacity to recycle damaged proteins is implicated as an acclimation response to UV-B damage in maize.

An oxidative burst can be a direct consequence of exposure to UV-B photons, and plants respond through a variety of anti-oxidative strategies. SOM c6 contains three different transcripts for cytochrome P450 proteins. In addition, both BZ1 glucosyl transferase and chalcone synthase targets are included in this group. Even if *b*, *pl* plants are deficient in B and Pl transcription factors, which regulate the expression of these two genes, a low level of expression could result if these genes are independently regulated by UV-B in leaves [27] or if cross-reacting transcript types are induced.

SOM cluster 9 includes transcripts downregulated by UV-B in shielded tissues, seedling roots, and immature ears. This cluster contains 34 ESTs, 13 of which have no match to any sequence in GenBank. It is interesting that members of this cluster with putative functions are genes involved in signal transduction (calmodulin and a calcium-dependent protein kinase), and one transcription factor (homologous to GATA-binding transactivating protein from *Arabidopsis*). Additionally, transcripts for both alpha and beta tubulins are

downregulated. These results illustrate that UV-B irradiation of adult leaves, under conditions in which photosynthesis is hardly perturbed (<10% reduction; P.C. and V.W., unpublished work), can profoundly affect distant organs.

Confirmation by RNA gel-blot analysis and real-time RT-PCR

To determine whether the transcript changes identified by microarray analysis are reliable, total RNA obtained from the same irradiated and control plants used for microarray experiments was examined by RNA gel-blot analysis (Figure 5). Three genes representing different SOM clusters (RuBisCO small subunit, SOM c0; ribosomal protein L11, SOM c4; and cinnamyl alcohol dehydrogenase, SOM c5) were selected as probes. The blot hybridization results correspond closely in magnitude and in the sensitivity of response to UV-B to the microarray results for these genes (Figure 5). For example, transcripts for RuBisCO small subunit are lower after UV-B exposure in adult leaves, but the levels of this transcript are unchanged in seedling leaves.

In addition, we did real-time reverse transcription PCR (RT-PCR) experiments to validate the microarray results for other transcripts that show differences after the UV-B treatments. This technique is both highly sensitive and accurate in quantifying transcript abundance; precise gene identification was achieved by sequencing the RT-PCR products. Table 1 shows a list of transcripts that are up- or downregulated by the 8-hour UV-B treatment in the microarray experiments, and a comparison with results obtained by northern blot or real-time RT-PCR. The values obtained from both methods correspond closely in magnitude to the microarray results for these genes, demonstrating that the microarray data are highly reproducible.

Seedling leaves have higher levels of a UV-absorbing compound than adult leaves

Because seedling leaves showed fewer transcript changes after UV-B radiation, they may possess greater shielding capacity than adult leaves. *b*, *pl* plants are deficient in anthocyanin, but they could contain other UV-B-absorbing molecules. Previously, we found that maize plants with different levels of anthocyanins also contain a methanol-extractable UV-absorbing molecule with a maximum absorbance in the UV-A region [24]. As described in Materials and methods, extracts were prepared and UV-A-absorbing compounds separated by high-performance liquid chromatography (HPLC). A main peak with a retention time of 17 min (data not shown) is increased by UV-B radiation in a dose-dependent manner (Figure 6a). The concentration of this molecule increases up to 10-fold after 8 hours irradiation at the intensity of 0.36 W/m² used for samples in the microarray analysis. Under identical HPLC conditions, samples from different leaf developmental stages grown at a UV-B fluence of 0.09 W/m² were also examined. As shown in Figure 6b, the concentration of the 17-min retention time molecule is about 12-fold higher in

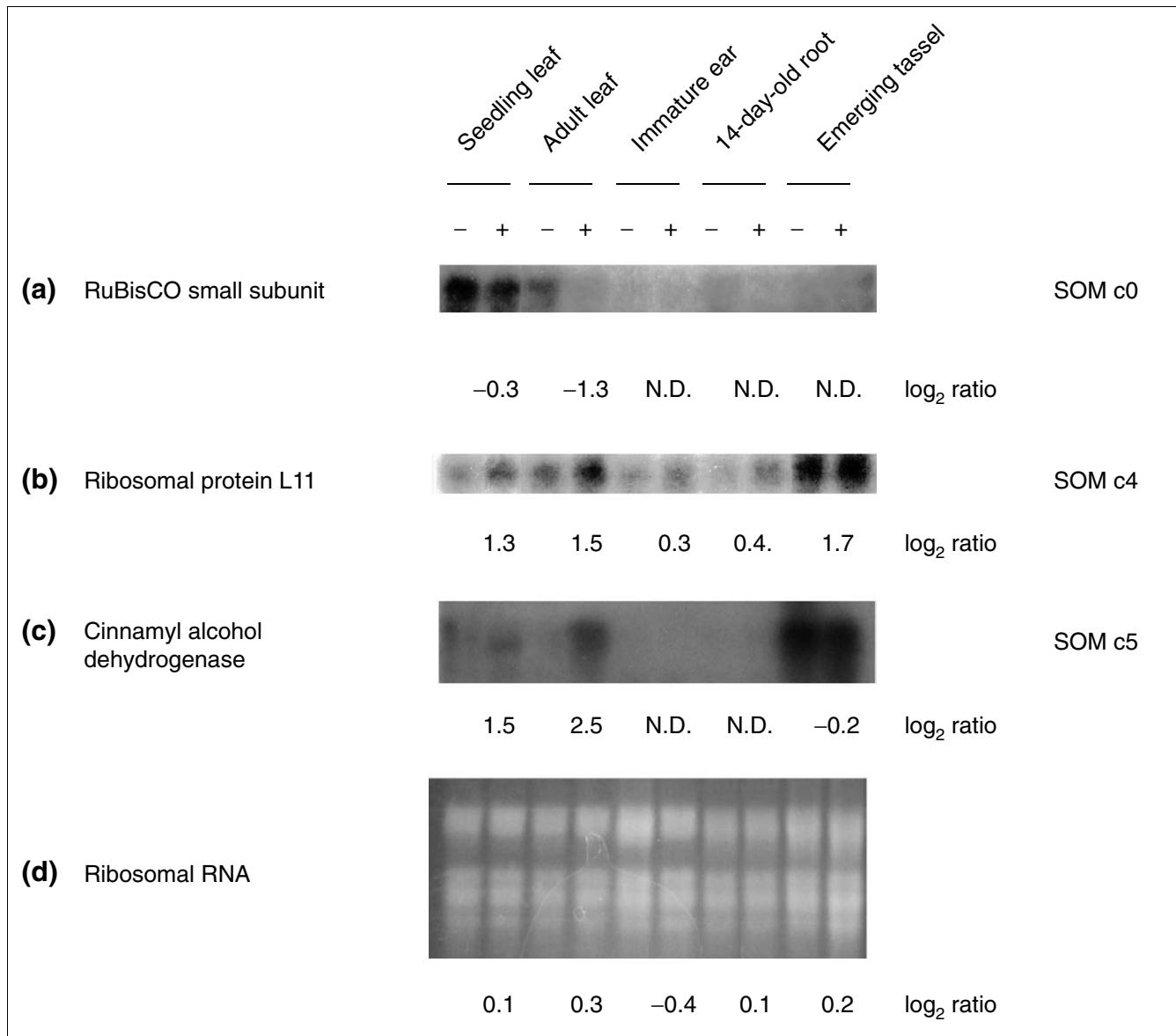


Figure 5
 RNA gel-blot analysis to confirm microarray data. Lanes contained 10 μg of total RNA extracted from the different tissues after UV-B (+) and no UV-B (-) treatments. Several identical gels were prepared and blotted. Each blot was hybridized with ³²P-labeled RuBisCO small subunit **(a)**, ribosomal protein L11 **(b)** or cinnamyl alcohol dehydrogenase **(c)** probes. **(d)** Ethidium-bromide-stained gel as a check for equal loading. The log₂ ratio was calculated as for microarray experiments by quantification of hybridization signals and ethidium-bromide-stained bands using Kodak ds ID Digital Science, as described in Materials and Methods. The log₂ ratio is provided at the bottom of each blot, using as a reference RNA from plants that were grown under natural levels of UV-B. ND means that the signal was too low for quantification.

seedling (leaves 1 to 5) compared to adult leaves (leaves 10-11), and the levels of this UV-absorbing molecule are intermediate in juvenile samples (leaves 6-9). The compound was purified after HPLC separation and the absorption spectrum is shown in Figure 6c. There are two major peaks of absorbance: the first is at 260 nm and the second at 345 nm, with substantial absorption in the UV-B range as well. This compound can therefore act as a natural UV protectant. Given its

high concentration in seedling leaves, it is a likely contributor to the observed higher tolerance of the initial leaves in a young plant to UV-B radiation. Other mechanisms of protection in seedling leaves cannot be ruled out. For example, cuticular waxes in maize are heavily deposited on juvenile tissues and could also protect the plant against UV-B [34]; seedling leaves might also have a different threshold for UV-B induced transcriptome changes.

Table 1**Confirmation of microarray data by northern blot and real-time RT-PCR assays**

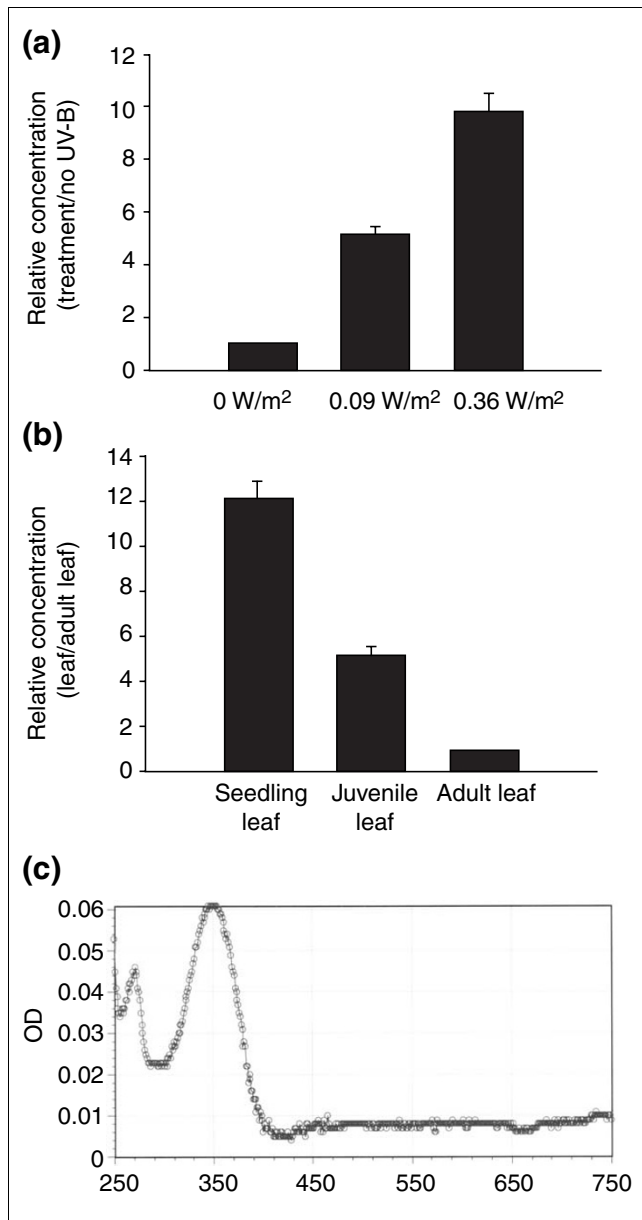
Description hit	GenBank accession number	Method used	Adult leaf	Seedling leaf	14-day-old root	Immature ear	Emerging tassel
RuBisCO small subunit	AI855224	Microarrays	<i>-1.22</i>	0.20	0.33	0.68	0.61
		Northern blot	<i>-1.30</i>	0.30	ND	ND	ND
Ribosomal protein L11, cytosolic	AI948309	Microarrays	1.09	1.05	0.28	0.54	1.45
		Northern blot	1.50	1.30	0.40	0.30	1.70
Cinnamyl alcohol dehydrogenase	AW927923	Microarrays	1.19	1.22	-0.63	F	0.40
		Northern blot	2.50	1.50	ND	ND	-0.20
Histone deacetylase	AW438666	Microarrays	1.07	-0.69	1.04	0.84	0.69
		Real-time RT-PCR	1.49	-0.29	1.09	ND	0.86
Cysteine proteinase	AW129800	Microarrays	1.08	0.19	0.09	F	0.51
		Real-time RT-PCR	3.19	0.89	0.82	ND	0.75
Methyl-binding protein	AW737448	Microarrays	F	1.31	1.03	-0.30	-0.11
		Real-time RT-PCR	0.79	1.11	1.07	ND	0.24
Cytosine 5' DNA methyltransferase	AW215926	Microarrays	1.75	0.02	0.79	-0.32	0.56
		Real-time RT-PCR	2.27	-0.88	-0.51	ND	0.46
Membrane protein Mlo5	BE025314	Microarrays	F	1.04	1.20	-0.25	-0.10
		Real-time RT-PCR	0.97	1.62	1.34	ND	0.42
snRNP Sm protein F	AW330881	Microarrays	2.72	1.84	1.02	<i>-1.06</i>	0.39
		Real-time RT-PCR	1.69	5.25	1.92	ND	0.71
	AW433427	Microarrays	3.04	1.03	<i>-1.29</i>	-0.99	0.21
		Real-time RT-PCR	2.12	1.21	<i>-1.07</i>	ND	0.79

The numbers correspond to the \log_2 ratios. The transcripts that are upregulated by UV-B by more than two-fold are in bold type, while transcripts downregulated by UV-B by more than two-fold are in italic. F, flagged ESTs which had signals similar to the background in some condition and were eliminated during the analysis; ND, not determined.

Effects of UV-B supplementation on gene expression in shielded leaves

To better understand the impact of UV-B in tissues not directly exposed to radiation, we examined the responses in shielded organs in more detail. For this purpose, two different experiments were carried out. In the first protocol, one adult leaf per plant was covered with a polyester plastic sheath that absorbs UV-B (PE, see Materials and methods). Another leaf on each plant was covered with a cellulose acetate plastic that allows UV-B transmittance (CA) as a control for differences in temperature and humidity inside the sheath. After an 8-hour UV-B treatment, transcripts from leaves covered with the two plastics were compared by microarray hybridization; the PE-

covered leaf should respond to UV radiation only if there is a signal transmitted from exposed leaves. In the second protocol, we compared transcripts from PE-covered leaves in plants exposed to UV-B to those from PE-covered leaves in unirradiated plants; only the PE-covered leaf on an irradiated plant should exhibit transcript changes. The results from both hybridization protocols were compared to the dataset for adult leaves for analysis. Of the 121 transcripts upregulated by UV-B in adult leaves (Figure 2), 48 were also upregulated in PE-covered leaves in UV-B irradiated plants in both protocols (see Additional data file 2). This strengthens the interpretation of the results presented in Figure 2 in which responses were detected in naturally shielded ears and roots.

**Figure 6**

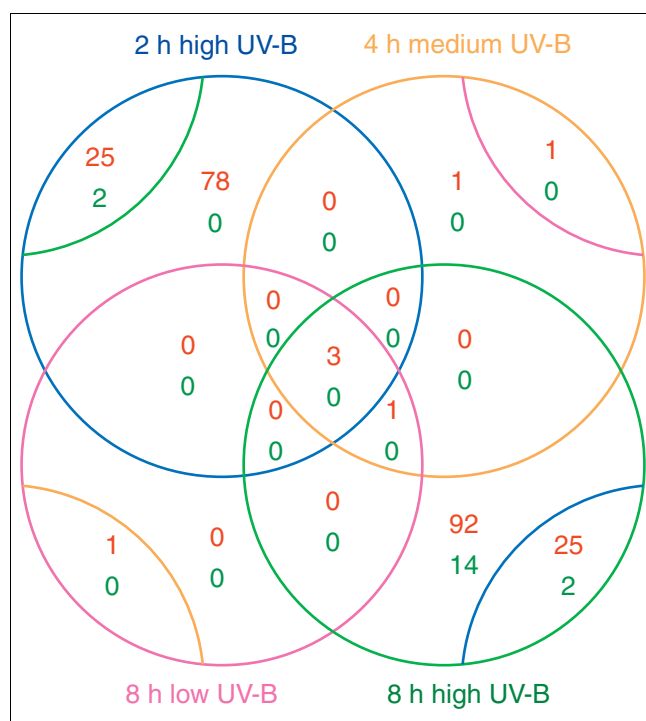
UV-absorbing pigment in maize leaves. **(a)** Increase in a UV-absorbing pigment after UV-B exposure. The concentration of the compound was determined by integration of the area of a peak with a retention time of 17 min (data not shown) after HPLC separation; this is expressed relative to the concentration of pigment in plants not treated with UV-B radiation. Error bars are standard errors. **(b)** UV-absorbing pigment in maize leaves at different developmental stages. The concentration of the compound was determined by integration of the area of a peak with a retention time of 17 min after HPLC separation; this is expressed relative to the concentration of pigment in adult plants at 0.09 W/m² UV-B. Error bars are standard errors. **(c)** Absorption spectrum in acidic methanol of the purified compound after HPLC separation. The spectrum is similar to that obtained with a number of non-anthocyanin flavonoids; it could be a single molecule or a mixture of molecules with similar properties in the HPLC assay.

We propose that a signal(s) must be transmitted from exposed to shielded organs, permitting indirect UV-B induction of some genes in the absence of direct exposure to UV-B and the consequent damage to DNA, RNA, and protein. It is important to note that 73 transcript types are upregulated in exposed leaves but not in PE-covered leaves; this subset probably represents direct responses to radiation or its immediate cellular consequences. Similarly, naturally shielded organs exhibit fewer transcript changes than do exposed organs (Figure 2).

Of the 48 ESTs differentially expressed in the shielded leaf, 21 have assigned putative functions that define several classes of response. One group contains a cytochrome P450 monooxygenase and two dioxygenases; enzymes encoded by such transcripts could be involved in detoxification of oxidized products generated by interaction with ROS. ROS moving from exposed tissues or produced locally in shielded tissues after detection of a signal(s) from irradiated leaves may be involved in the propagation of UV-B stress signals to shielded tissues. Two RAD proteins are also induced in shielded leaves; one is RAD17, which, as described above, is involved in activation of DNA replication checkpoints [33]. RAD6 is a ubiquitin-binding enzyme that also participates in post-replication repair of DNA in yeast [35]. Even though direct DNA damage does not occur in shielded organs, it appears that the regulators of cell-cycle progression are modulated there as a response to an unknown signal from irradiated tissues. A third gene type upregulated in shielded leaves encodes a sphingosine-1-phosphate lyase (GenBank AI855283). This enzyme is involved in degradation of sphingosine 1-phosphate, a polar sphingolipid metabolite that has been proposed to act both as an extracellular mediator and as an intracellular second messenger [36]. Extracellular effects are mediated via a recently identified family of plasma membrane G-protein-coupled receptors in mammalian cells, whereas specific intracellular sites of action remain to be defined [36]. Sphingosine 1-phosphate is thus a candidate molecule participating in UV-B signaling, as it is also involved in signaling in plants [37]. Genes for protein degradation are also upregulated in UV-B-shielded leaves. Finally, several transcripts associated with stress responses are listed in Additional data file 2, such as a salt stress-induced protein and a thaumatin; these results indicate that shielded tissues may experience physiological changes after UV-B damage has occurred elsewhere in the plant.

Transcription in leaves is affected by fluence rate independently of the total dose

To test if transcripts regulated by UV-B in adult leaves exhibit reciprocity (duration × intensity = response) or a threshold-type response, a total effective dose of UV-B corresponding to 2.25 kJ/m²/day normalized to 300 nm was administered to different adult plants for 2 hours (high UV-B irradiance, 0.36 W/m²), for 4 hours (medium UV-B irradiance, 0.18 W/m²), or for 8 hours (low UV-B irradiance, 0.09 W/m²). As a control

**Figure 7**

Venn diagram comparisons between genes regulated by UV-B under different irradiation and/or total doses in adult leaves of maize.

Upregulated genes are colored red, downregulated genes green. Sets of genes were selected using the criteria described in Materials and methods. In blue: transcripts regulated by high levels of UV-B (0.36 W/m^2) during 2 h; in orange: transcripts regulated by medium levels of UV-B (0.18 W/m^2) during 4 h; in pink: transcripts regulated by low levels of UV-B (0.09 W/m^2) during 8 h; in green: transcripts regulated by low levels of UV-B (0.36 W/m^2) during 8 h.

for circadian effects on gene expression, samples were collected from control (no UV-B) plants at the same times. Transcript levels were compared in microarray experiments that examined each UV-B-treated sample compared to the control. Although many plant responses to radiation exhibit reciprocity, this relationship did not hold for most transcripts examined in our experimental conditions. As shown in Figure 7, 106 transcripts were induced after 2 hours of high UV-B, while only six were upregulated after 4 hours of medium-fluence UV-B, and only five after 8 hours at low UV-B irradiance. Interestingly, only two ESTs were downregulated by UV-B in the 2-hour, high-fluence UV-B treatment, and none in the longer-exposure, lower-irradiance treatments. These results indicate that there is a threshold of irradiance intensity for the elicitation of most maize responses in adult leaves.

Using the highest irradiance (0.36 W/m^2), two total dosages (2 hours ($2.25 \text{ kJ/m}^2/\text{day}$) and 8 hours ($9 \text{ kJ/m}^2/\text{day}$)) were compared in adult leaf samples. More transcripts showed a greater than twofold difference to expression in control samples after the longer duration and hence higher total dose of UV-B (108 after 2 hours compared to 137 after 8 hours).

Transcripts could be classified as rapid, transitory responses (78 transcripts altered at 2 hours but similar to the control at 8 hours), rapid but persistent responses (30 transcripts), and delayed responses (107 transcripts similar to control at 2 hours but altered at 8 hours). After 2 hours of high irradiance, the rapid but transitory responses include three genes with putative functions assigned: a receptor protein kinase, GenBank AW433410; a potassium transporter, AI947597; and ADP-glucose pyrophosphorylase large subunit, AW438209. The last gene is also UV-B induced after 8 hours UV-B exposure in seedling leaves and roots (see Additional data file 1). During a 2-hour treatment, no transcript types were downregulated at the more than twofold change criterion. The rapid, persistent responses include 27 ESTs that have no match to any other in GenBank (data not shown). The three ESTs with assigned functions are an F_1 -ATPase alpha subunit, GenBank AW191100 and two genes of the anthocyanin biosynthetic pathway, *bz1* and a chalcone synthase. The latter two genes are also UV-B upregulated by the low- and medium-intensity UV-B treatments (intersection of all treatments, Figure 7) and in seedling leaves after 8 hours UV-B exposure (see Additional data file 1), indicating that they have a lower threshold of UV-B perception for induction. The delayed UV-B responses transcript types include 92 upregulated and 14 downregulated ESTs. Interestingly, transcripts for photosynthetic enzymes (such as RuBisCO small subunit, a PSII 22 kDa polypeptide and a PSI P700 apoprotein A2) are only downregulated after 8 hours of high-irradiance UV-B and not by lower dosages or by a 2-hour high-irradiance exposure. The results from experiments manipulating dosage and duration collectively indicate that there are thresholds for nearly all gene responses for both treatment length and radiation intensity.

Kinetics of UV-B effects on gene expression using RNA gel blots and real-time RT-PCR

RNA blot hybridization and real-time RT-PCR were used to analyze the kinetics of UV-B transcript changes in both directly exposed (adult leaf) and shielded (root) tissues. For experiments using adult leaves, two cDNAs that were upregulated within 8 hours in this organ were utilized as probes for northern blots. In the first protocol to determine when transcripts are induced, adult leaves were exposed under UV-B lamps for 2, 4, 6 and 8 hours at 0.36 W/m^2 ; samples were collected immediately after the UV-B treatment from irradiated and control plants. As shown in Figure 8, a 2-hour UV-B exposure suffices to increase transcript levels of clathrin (GenBank AW134461) and ribosomal protein L11 (AI948309), although the increase is lower than the twofold cut-off in the microarray experiments (see Additional data file 1). Clathrin transcripts (Figure 8a) show a progressive increase with longer exposures; in contrast, ribosomal protein L11 transcripts are approximately equivalent at 2 and 8 hours. In the second protocol to explore the persistence of transcript upregulation in the absence of UV-B, leaves were UV-B-irradiated for 2, 4 or 6 hours, followed by a period

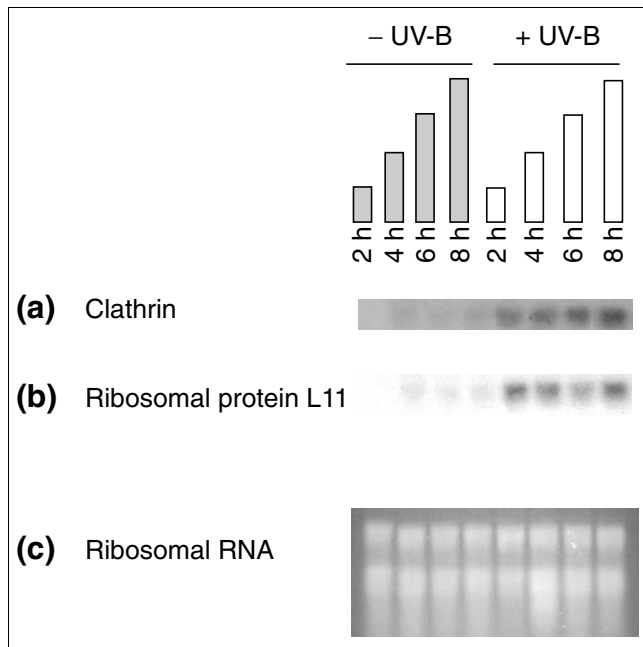


Figure 8
RNA gel-blot analysis to study the kinetics of UV-B induction of gene expression in adult leaves under experimental protocol 1. Lanes contained 10 µg of total RNA extracted from adult leaves after 2, 4, 6 and 8 h of UV-B (+) and no UV-B (-) treatments. Several identical gels were prepared and blotted. Each blot was hybridized with ³²P-labeled clathrin (a) or ribosomal protein L11 (b) probes. (c) Ethidium-bromide-stained gel as a check for equal loading. Bars in gray indicate light treatment without UV-B; bars in white indicate light treatment with UV-B supplementation for the time indicated in the figure.

without UV-B to complete an 8-hour treatment; samples were also collected from plants irradiated for 8 hours followed by a 12-, 24- or 48-hour recovery period. As shown in Figure 9, clathrin and ribosomal protein L11 transcripts are induced with exposures from 2 to 8 hours, as expected from the initial experiment. For clathrin, a short exposure of UV-B is enough to upregulate this gene, but a longer time is required to reach higher levels of expression. Both transcripts persist in recovery periods of 2 hours (after a 6-hour exposure), 6 hours (after a 2-hour exposure), and 12 hours (after an 8-hour exposure). After 12 hours without UV-B, transcripts are lower than at the end of the UV-B treatment, and after 24 hours transcript levels are similar to the non-irradiated control plants. The same RNA samples used in the experiments above were used to compare UV-B-regulated expression of other genes by real-time RT-PCR. Table 2 shows that, as observed for clathrin transcripts, UV-B induction of a cysteine proteinase (GenBank AW129800) shows a progressive increase with longer exposures, while transcripts for a histone deacetylase (AW438666) and for a cytosine 5' DNA methyltransferase (AW215926) are approximately equivalent at 2 and 8 hours.

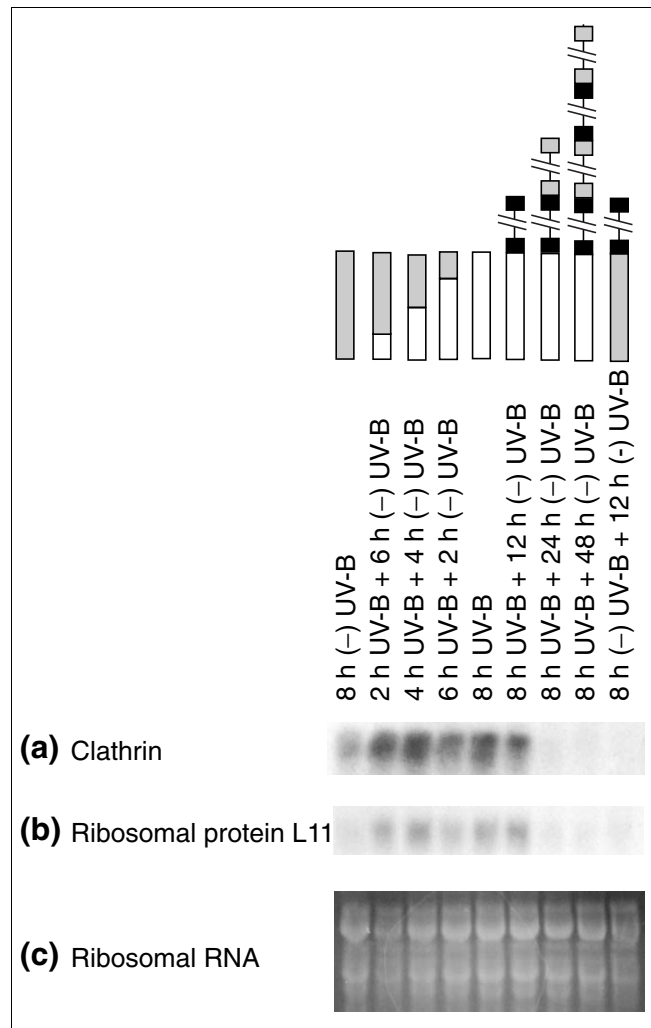


Figure 9
RNA gel-blot analysis to study the kinetics of UV-B induction of gene expression in adult leaves under experimental protocol 2. Lanes contained 10 µg total RNA extracted from adult leaves after 2, 4, 6 or 8 h of UV-B (+) followed by a no UV-B period to complete 8 h, and after 8 h of UV-B followed to a period of 12, 24 or 48 h of no UV-B (+), and from no UV-B (-) treatments. Several identical gels were prepared and blotted. Each blot was hybridized with ³²P-labeled clathrin (a) or ribosomal protein L11 (b) probes. (c) Ethidium-bromide-stained gel as a check for equal loading. Bars in gray indicate light treatment without UV-B; bars in white indicate light treatment with UV-B supplementation; and bars in black indicate dark treatment for the time indicated in the figure.

Blot analysis using RNA from roots of 14-day-old seedlings was also performed after 2, 4, 6, or 8 hours of plant irradiation; as shown in Figure 10, probes were cDNAs from transcripts detected to be upregulated in roots in UV-B plants (a membrane protein M105, GenBank accession number BE025314; a receptor kinase-like protein, GenBank accession number BE128804; and a transmembrane protein, GenBank accession number AW313343). Samples from non UV-B-irradiated plants were collected at the same periods of time as

Table 2**Comparison of the kinetics of UV-B induction of gene expression in adult leaves by real-time RT-PCR**

Description hit	GenBank accession number	Treatment	2 h UV-B	4 h UV-B	6 h UV-B	8 h UV-B
Histone deacetylase	AW438666	(1)	1.09 ± 0.03	1.17 ± 0.03	1.24 ± 0.04	1.49 ± 0.05
		(2)	1.29 ± 0.07	1.49 ± 0.02	1.09 ± 0.02	1.49 ± 0.05
Cysteine proteinase	AW129800	(1)	1.09 ± 0.05	1.26 ± 0.07	2.09 ± 0.03	3.19 ± 0.09
		(2)	3.10 ± 0.08	2.89 ± 0.08	3.32 ± 0.05	3.19 ± 0.09
Cytosine 5' DNA methyltransferase	AW215926	(1)	1.75 ± 0.07	2.43 ± 0.03	1.89 ± 0.04	2.27 ± 0.12
		(2)	2.27 ± 0.11	1.87 ± 0.09	2.12 ± 0.07	2.27 ± 0.12

The numbers correspond to the log₂ ratios. Experiments were done at least in triplicate. Adult leaves were exposed under UV-B lamps for 2, 4, 6 and 8 h at 0.36 W/m², and samples were either collected immediately after the UV-B treatment (1) or after a period without UV-B to complete an 8-h treatment (2).

controls. UV-B regulation of all three genes is very rapid, because high transcript levels are apparent after 2 hours (Figure 9). Transcript levels of all three genes are further increased with longer times of UV-B exposure of the leaves.

For finer resolution of the time required for gene induction, real-time RT-PCR assays were established for four additional genes. By microarray hybridization a histone deacetylase was strongly induced by an 8-hour UV-B treatment in both roots and adult leaves, methyl-binding protein was induced more than twofold in roots but unchanged in leaves, RAD5 was unchanged in roots, and cytosine 5' DNA methyl transferase was upregulated in leaves but not roots (see Additional data file 1). For the four genes tested, none was significantly upregulated by 30 min of UV-B exposure; however, histone deacetylase transcript abundance was downregulated four- to eightfold in 30 min (Figure 11). Within 60 min, histone deacetylase levels had increased fourfold above those in the non-irradiated control roots and by 90 min irradiated leaves also had a fourfold increase in this transcript type. The unusual behavior of histone deacetylase has been verified in three repetitions of the real-time PCR assays using independent biological samples. By 60 min, methyl transferase in leaves and methyl-binding protein transcripts in roots were also induced (Figure 11). RAD5 remained constant in roots and methyl-binding protein transcripts remained constant in leaves over the 90 min exposure period, confirming the microarray hybridization results in which these transcripts remain unchanged in plants exposed to UV-B for 2 to 8 hours. The time-course experiments with nine genes collectively indicate that UV-B can modulate transcript abundance very quickly, even in roots where the signal(s) mediating UV-B effects must be produced and translocated from directly exposed tissues.

Discussion

Of 5,664 maize transcript types examined from the anthocyanin-deficient *b*, *pl* line by microarray hybridization, 347 are regulated by an 8-hour UV-B-exposure in at least one of five organs. Interestingly, most of the UV-B-regulated genes are organ-specific. A previous report established that individual maize organs express discrete suites of genes [38], and we find that responses to UV-B also reflect cellular differentiation. One hundred and eight transcript types are induced in adult leaves within a 2-hour exposure period, and a subset of the altered transcript levels persists during an 8-hour exposure; genes with a delayed response were also identified. By monitoring nine selected transcripts using RNA blot or real-time RT-PCR we confirmed that the activation of gene expression by UV-B radiation can be very rapid, both in directly exposed and shielded tissues - some transcripts show high levels of induction after only 1 or 2 hours of UV-B exposure. For these genes, transcript levels remain high for some time after UV-B illumination is finished, but return to basal levels one day later.

Shielded leaves as well as roots and immature ears receive a signal(s) from irradiated tissues that triggers numerous transcriptome changes. In roots and ears the major response is downregulation of transcript abundances. Similarly shielded leaves on an irradiated plant exhibit many transcriptome changes. These new findings that shielded leaves and organs respond rapidly indicates that UV-B induces an extensive, large-scale integrated response as summarized in Figure 12. The magnitude and diversity of responses is similar to what has been documented as systemic responses after pathogen attacks in a restricted location [39]. Future studies will be directed to define the mechanisms activating UV-B-responsive genes not only in directly exposed but also in shielded

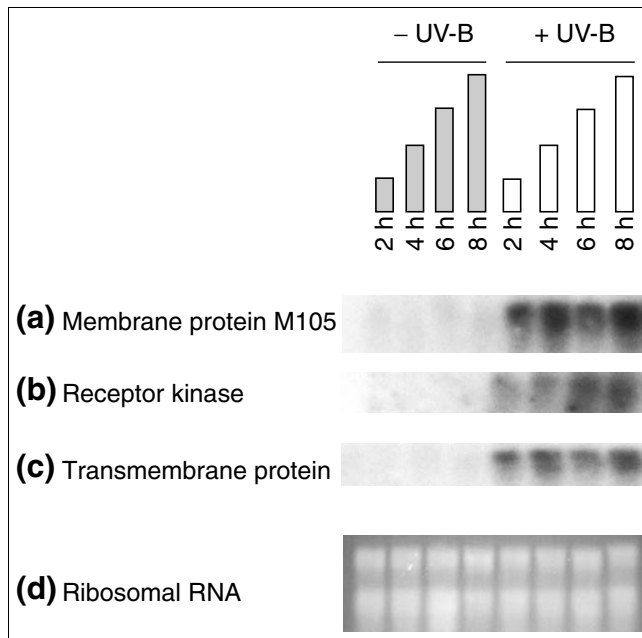


Figure 10
 RNA gel-blot analysis to study the kinetics of UV-B induction of gene expression in 14-day-old roots. Lanes contained 10 µg total RNA extracted from roots after 2, 4, 6 or 8 h of UV-B (+) and no UV-B (-) treatments. Several identical gels were prepared and blotted. Each blot was hybridized with ³²P-labeled membrane protein M105 (a), receptor kinase (b) or transmembrane protein (c) probes. (d) Ethidium-bromide-stained gel as a check for equal loading. Bars in gray indicate light treatment without UV-B; bars in white indicate light treatment with UV-B supplementation for the time indicated in the figure.

tissues and to identify the signals that activate distinctive gene expression in individual organs.

Among 347 transcripts found to be regulated by an 8-hour UV-B treatment, 285 showed upregulation in at least one organ while only 80 were downregulated by UV-B (Figure 2). For 18 genes, transcripts were upregulated by UV-B in some tissues but decreased in others, particularly in roots. The reason for this observation remains to be investigated after identification of gene function. Further experiments will be required to determine if this regulation occurs through gene expression control via different *cis*-acting promoter (to alter transcription) or RNA structural elements (to alter RNA half-life) or through convergence of distinct signal transduction pathways to act on the same element to produce up- or down-regulation. A limitation of currently available maize microarrays is that cDNAs are the spotted elements; therefore, determination of which members of cross-hybridizing gene families respond to UV-B treatment awaits gene-specific methods such as oligonucleotide arrays. In the short term, verification experiments with gene-specific real-time RT-PCR primers can selectively verify microarray results. As documented for nine examples of transcripts with different expression patterns, the verification experiments confirmed

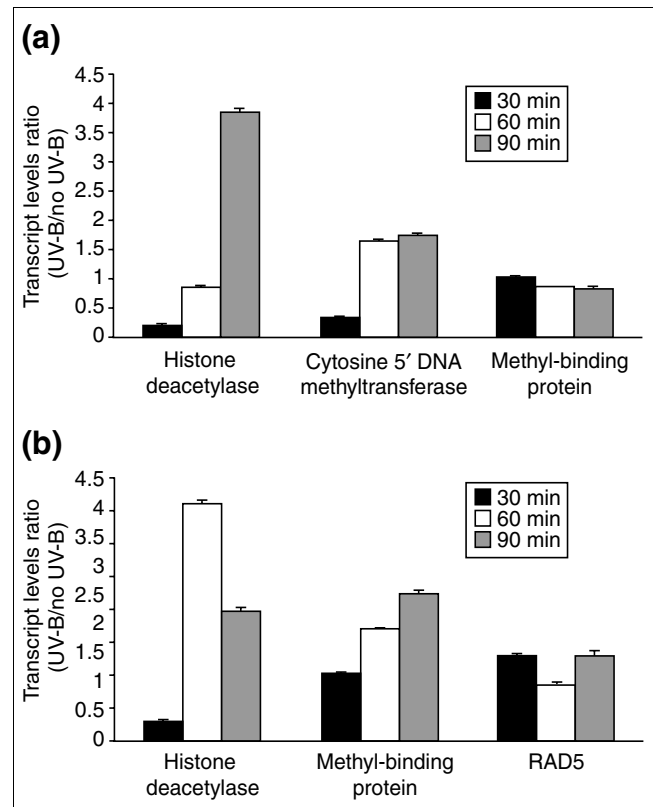
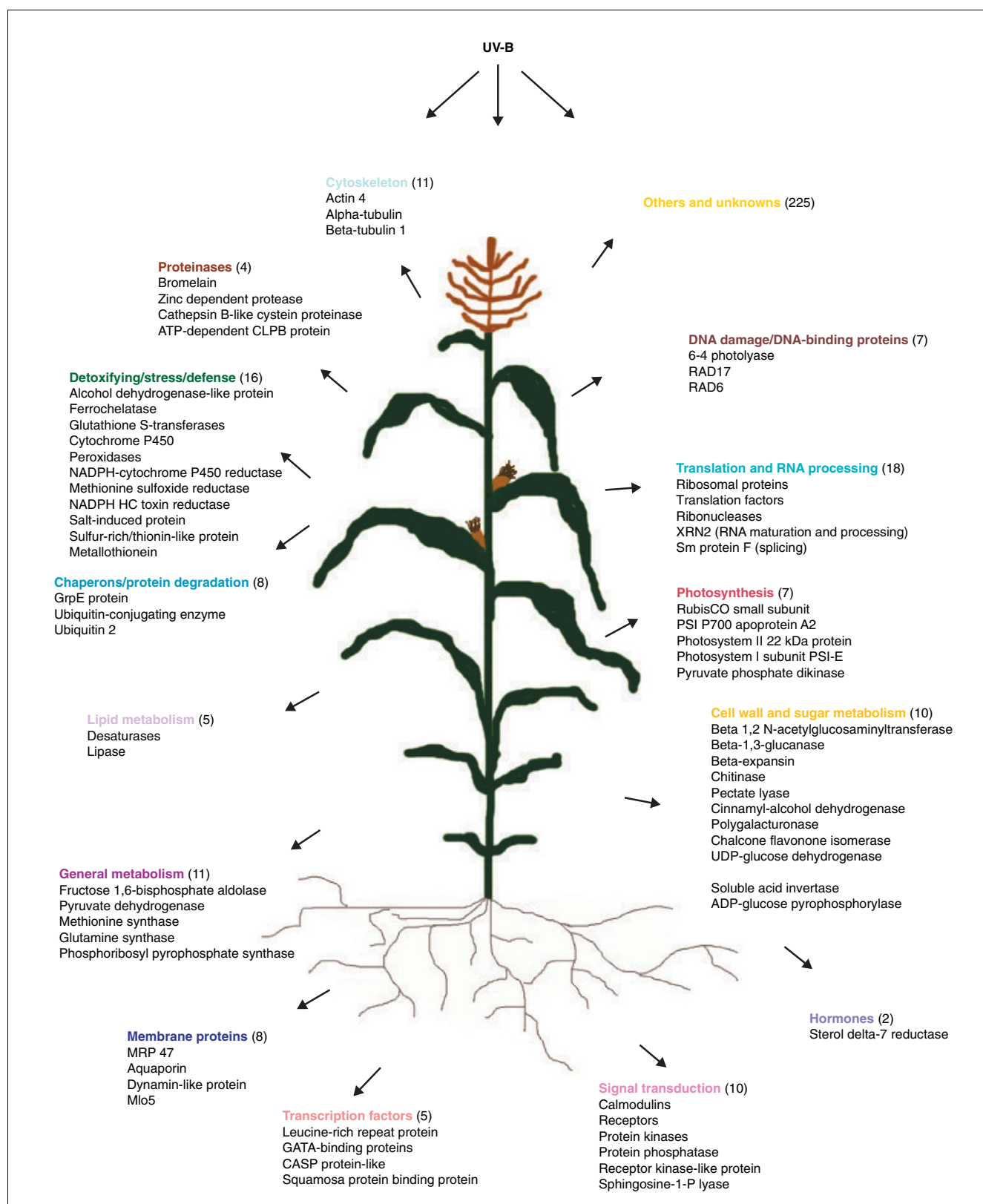


Figure 11
 Real-time RT-PCR analysis to study the kinetics of UV-B induction of gene expression in adult leaves and 14-day-old roots. cDNA (50 ng) obtained by reverse transcription of RNA from (a) adult leaves and (b) roots after 30 min, 60 min and 90 min of UV-B and no UV-B treatments was used for real-time PCR. Experiments were done at least in triplicate. Error bars are standard errors.

the conclusions drawn from the microarray hybridization results.

The plant's perception of UV-B that results in transcript abundance changes does not exhibit reciprocity: there is a dosage threshold for most genes. Adult tissues (leaves and tassel) that are directly exposed to supplementary UV-B exhibit more changes in gene expression than do seedlings or shielded organs such as roots and immature ears. Photosynthesis-associated genes are also unaffected in seedling leaves. We found that seedling leaves have higher levels of a UV-absorbing compound than adult leaves and that this compound is also induced by UV-B radiation. Thus seedling leaves are better shielded than adult leaves against UV-B. Seedling leaves may also be protected against UV-B by a waxy coating that can attenuate its impact [34] or may require either a higher intensity or duration threshold to trigger a response. Extending previous observations by including more genes involved in specific responses, we confirmed that downregulation of photosynthetic genes and induction of ribosomal protein genes occur after UV-B exposure in irradiated

**Figure 12**

Classification of UV-B-regulated genes identified by microarrays on the basis of their putative function. Transcripts with ratios at least twofold enhanced or decreased after the various UV-B treatments were included in the diagram.

adult leaves and now report that these changes do not occur in leaves sheathed in UV-B-absorbing plastic. The genes that respond after direct UV-B radiation include many examples of those involved in damage control. On a longer time scale, plant morphology depends on the patterns of cell proliferation and the direction and extent of subsequent cell expansion. UV-B modulates plant morphology, and the data reported here, in which regulators of the cell cycle are altered in shielded organs, could explain these observations. Both the immature ear and the root system contain zones of cells that are proliferating rapidly.

Specificity of the UV-B response

Although UV-B can trigger production of ROS, and these molecules can in turn stimulate signal transduction cascades, the specificity of maize responses to UV-B compared to other environmental perturbations [24] that elicit ROS requires that some aspect of the mechanism be restricted to UV-B perception. The best-documented specific outcome is UV-B damage to DNA. Our results, particularly documenting the profound changes in gene expression in shielded organs, indicate that UV-B elicits a range of responses in addition to the well-documented DNA damage and subsequent repair. It is possible that UV-B photons directly affect a key regulatory protein in irradiated cells. From our data, an enhanced capacity to repair and recycle damaged proteins can be implicated as an acclimation response to UV-B in most maize tissues. It is also possible that the combination of UV-B-induced damage to DNA, RNA, proteins and lipids, plus ROS, channels plant responses into a specific mode.

Long distance signal

We propose that signals from UV-B-irradiated tissues move rapidly to shielded organs, where they trigger physiological changes in the recipient cells. Consequences of signal perception include downregulation of diverse transcript types. Post-translational regulation may be an important response after UV-B in these tissues, as we found significant changes in the transcript levels of ubiquitin, ubiquitin-binding proteins and proteinases in shielded tissues. A key question concerns the nature of the signals within irradiated organs that trigger transcript abundance changes and the signal(s) produced in irradiated cells that elicit rapid transcriptome changes in distant shielded organs. At the dosages used, UV-B has little impact on photosynthesis in maize (<10% decrease compared with controls after 8 h exposure; P.C. and V.W., unpublished work). The types of transcripts changed by UV-B in shielded tissues (see Additional data files 1 and 2) include some genes involved in signal transduction and transcripts for membrane receptors. Transcripts for a sphingosine-1-phosphate lyase are upregulated in shielded leaves; the sphingolipid could act either as an extracellular mediator or as an intracellular second messenger in UV-B responses [37].

Integrative role for hydrogen peroxide

Previous studies have shown that UV-B exposure increases ROS species generating oxidative stress in irradiated organs [40,41]. It was proposed that in response to UV-B radiation ROS function as destructive radicals and may also be components of signal cascades that change plant gene expression [19]. ROS are putative candidates for signal molecules that could be involved in UV-B responses, particularly as we found that transcripts for enzymes involved in oxidative stress and detoxification were upregulated in shielded leaves covered with UV-B absorbing plastic (such as cytochrome P450 and metallothionein; see Additional data file 2) only when the rest of the plant was irradiated with UV-B. The role of reactive oxygen species, especially H_2O_2 , in integrating plant responses to biotic and abiotic stresses has been the focus of much attention. Hydrogen peroxide has been postulated to play multiple roles in plant defense against pathogens; among these are triggering programmed local cell death during the hypersensitive response, inducing defense genes near the site of infection, and acting as a signal in the induction of systemic acquired resistance [42]. It is thus feasible that H_2O_2 could also be involved in UV-B signaling within irradiated tissues as well as triggering responses in tissues not directly exposed to UV-B.

Figure 12 summarizes the impacts of UV-B on transcript regulation in the organs studied, combining genes of related function into major categories. In our previous study using microarray slides with fewer genes and RNA samples from maize leaves with different levels of flavonoids [24], the categories of photosynthetic proteins, ribosomal proteins, and enzymes involved in stress and cellular detoxification were shown to be affected by UV-B. A new group included in this work is proteins involved in DNA damage and DNA binding. This group of genes is only upregulated by UV-B in directly exposed tissues (see Additional data file 1). Cyclobutane pyrimidine dimers between adjacent pyrimidine bases as well as pyrimidine (6-4) pyrimidone dimers are formed after DNA absorbs UV-B photons. Genetic analysis has demonstrated that functional DNA repair pathways are essential for plant survival in UV-B, and because photosynthetic tissues are continuously exposed to UV-B in sunlight, these DNA repair pathways have been considered to be constitutively expressed [9]. In maize, transcripts for one 6-4 photolyase are upregulated by UV-B in emerging tassels (SOM co, see Additional data file 1). Moreover, transcripts for RAD6 are induced in tassels and adult leaves, while RAD17 is induced in seedling and adult leaves. These proteins are involved in post-replication repair of DNA in yeast, and they activate checkpoints that delay cell-cycle progression in yeast and human cells, respectively [33,35]. Our data suggest that constitutive expression may be an adaptive feature, but that, in addition, maize can acclimate to increased UV-B fluence by inducing some components of DNA repair.

Conclusions

We show here that direct exposure to UV-B results in significant upregulation (relative to non-UV-B-irradiated control) of many more transcripts than are downregulated. Most of the UV-B-regulated genes are organ-specific. Shielded organs experience little or no direct UV-B, but nonetheless display transcriptome alterations; organs directly exposed to UV-B probably produce signals that are transmitted to shielded organs where they elicit distinct transcriptome changes, primarily decreases in transcript abundance. These results indicate that there are integrated responses to supplemental UV-B. Collectively, the results from experiments manipulating dosage and duration indicate that there are thresholds for nearly all gene responses for both treatment length and radiation intensity. Transcriptome profiling after UV-B irradiation highlights possible signaling pathways and molecules for future research. An important next step is understanding the regulatory networks that permit such acclimation responses to UV-B and the relationship of UV-B stress to other abiotic challenges that plants cope with successfully.

Materials and methods

Plant material

The *b, pl* W23 line is maintained as a laboratory stock by self-pollination. This line is deficient in flavonoid accumulation (for details, see [24]).

Radiation treatments and measurements

UV treatments were carried out in a greenhouse illuminated for 14 h daily with a combination of sodium vapor, metal halide, and UV-A-containing bulbs to a fluence approximately 10% of noon summer white sunlight. At specific stages of development, plants were illuminated using UV-B lamps for 8 h (Phillips, F40UVB 40 W and TL 20 W/12) using fixtures mounted 30 cm above the plants with a biologically effective UV irradiance of 0.36 W/m² (9 kJ/m²/day) normalized to 300 nm [29]. As a comparison, the irradiation protocol used corresponds to the 'supplementation treatment' in [24]. This treatment was chosen because it provides more controlled conditions than experiments in the field, even if some responses may be different in this condition; and also because some experiments, such as shielding tassels in adult plants, are very difficult to do in the field because of the size of the fully developed maize plants. This UV-B flux rate corresponds to UV-B on 21 June at 50° from the equator at sea level with a 33% reduction from normal ozone levels. The bulbs were covered with cellulose acetate filters (CA, 100 mm extra-clear cellulose acetate plastic, Tap Plastics, Mountain View, CA); the CA sheeting does not remove any UV-B radiation from the spectrum but excludes wavelengths lower than 280 nm (UV-C). As a control of no UV-B, plants were exposed for the same period of time under the same lamps covered with polyester filters (PE, 100 mm clear polyester plastic; Tap Plastics). This PE filter absorbs UV-B. The output of the UV-B source and

other spectral data were recorded using an Optronics model 752 spectroradiometer (Optronics Laboratories, Orlando, FL) that was calibrated against a National Bureau of Standards certified radiation source before each use. The spectrum under each treatment was recorded periodically with 1 nm resolution across the entire sunlight spectrum (290 to 800 nm). After 8-h UV-B treatment, seedling or adult leaves, 14-day-old roots, emerging tassels or immature ears were collected from multiple plants for RNA extraction. Pooled samples from the same treatment regime reduce the variability compared to use of single individuals. The biological experiment was repeated at least twice.

For experiments to investigate the impact of UV-B in shielded leaves, leaf 9 or 10 in adult plants was covered with a plastic bag fabricated from either UV-B-absorbing PE or UV-B-transparent CA sheeting. Plants with one leaf covered by CA or PE were illuminated using UV-B lamps for 8 h; samples were collected for RNA extraction immediately after the end of the radiation treatment. As a second control for changes in temperature or humidity inside the plastic bags, leaf 9 or 10 from adult plants was covered with a PE plastic bag, and half of the plants irradiated with UV-B. Leaf temperature and humidity were recorded using an infrared thermometer (Model 210ALCS microcomputer-based agri-term infrared thermometer, Everest Interscience, Fullerton, CA) and a relative humidity hygrometer (Thermo-Hygro 800016, Sper Scientific, Scottsdale, AZ). Average leaf temperatures covered by the plastic bags were always within $\pm 0.5^{\circ}\text{C}$ of each other, and in no case were consistent differences in temperature detected in leaves covered by the different plastics; relative humidity differences were less than 25% between treatments.

To study reciprocity in the UV-B response, a total effective dose of UV-B normalized to 300 nm corresponding to 2.25 kJ/m²/day was administered to different adult leaves for 2 h (irradiance 0.36 W/m²), for 4 h (irradiance 0.18 W/m²) and for 8 h (irradiance 0.09 W/m²). Different irradiances were adjusted by placing the plants at different distances from the UV-B bulbs. As a control for circadian effects, samples were collected from no UV-B-irradiated plants at the same times. For experiments to study the kinetics of UV-B alteration of gene expression, plants were grown as described above and exposed under UV-B lamps for 30 min, 60 min, 90 min, 2, 4, 6 or 8 h. Leaf and root samples were collected for RNA extraction immediately after the end of the treatment or after the end of an 8-h treatment without UV-B. As controls, samples from untreated plants were also collected. Leaf samples were also collected from UV-B-exposed plants 8 h after the beginning of each treatment. Finally, plants were exposed for 8 h under UV-B light, and leaf samples were collected after 12, 24 and 48 h of the end of the radiation treatment. As control, leaf samples were collected simultaneously from plants not irradiated with UV-B.

RNA isolation, mRNA purification and probe synthesis

Multiple leaves and tissues from different plants from each radiation treatment were collected for RNA extraction. Because of the sensitivity of microarrays, plant-to-plant variation was reduced by bulk harvesting at least six samples of each tissue from different plants collected from each experimental treatment. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' recommendations. Poly(A) RNA was isolated using Oligotex (Qiagen, Valencia, CA), and 4 µg poly (A) RNA was used for each cDNA synthesis using Superscript II reverse transcriptase (Life Technologies, Carlsbad, CA). cDNA was labeled using 100 µM Cy5-dUTP or Cy3-dUTP (Amersham, Piscataway, NJ). Excess nucleotides and primers were removed using QIAquick PCR Purification Kit (Qiagen).

RNA gel blot hybridization

Total RNA (10 µg) was analyzed by electrophoresis on a 2% (v/v) formaldehyde/1.5% (w/v) agarose gels and blotted onto Hybond-N+ nylon membrane (Amersham). DNA probes were labeled with $\alpha^{32}\text{P}$ -dCTP by the random primer method and purified from unincorporated nucleotides using probe purification columns (Amersham). Hybridizations were done overnight at 42°C using 50% (v/v) formamide. Hybridization signals were quantified using Kodak ds 1D Digital Science (Scientific Imaging System, New Haven, CT).

Microarray experiments

Maize Unigene I arrays fabricated by the Maize Gene Discovery Project contain 5,664 ESTs [28]. About 90% of the spotted cDNAs showed significant hybridization when leaf mRNA was used for the experiments. Within these arrays, the cDNA samples are printed three times next to each other; consequently, average signal intensities and the ratio between co-hybridized samples could be assessed multiple times within each microarray and within experiments. These arrays contain cDNAs recovered from libraries of mixed adult tissues (707 and 945), mixed stages of embryo development (687), and salt-stressed roots (603). The experimental and reference samples were labeled with either Cy5-dUTP or Cy3-dUTP fluorescent dye (Amersham). Two samples, each labeled with one of the dyes, were mixed and then hybridized to a microarray for 15 h at 60°C. The slides were washed and then scanned with a GenePix 4000B Scanner (Axon Instruments, Union City, CA). Normalization between the Cy3 and Cy5 fluorescent dye-emission channels was achieved by adjusting the levels of both image intensities. The experiments were repeated at least twice with samples from different experiments as biological replicates. In these dye-swapping experiments, the RNA samples from different experiments were labeled reciprocally, both as a biological and technical repetition for comparing the reproducibility of the experiments.

Data analysis

The hybridization intensities of each microarray element were measured using Scanalyze 4.24 (available at [43]). The

two channels were normalized in log space using the z-score normalization on a 95% trimmed dataset. We removed unreliable spots according to the following criteria: spots flagged as having false intensity caused by dust or background on the array were removed, and spots for which intensity was less than threefold above background were also eliminated. Signals from triplicate spots were averaged. The data was submitted to the GEO repository, and the GEO Accession number of the series is GSE671. Multiple experiments were analyzed using Cluster and Treeview software [44]. To interpret the data, genes were grouped according to similarity of expression patterns by two algorithms. A hierarchical clustergram of genes was grouped by both related regulation patterns and expression amplitudes; and expression profiles were organized with self-organizing maps or SOMs. For hierarchical cluster analysis, we used the default options of hierarchical clustering using the uncentered correlation similarity metric. We performed the analysis using both normalized and non-normalized data; the outcomes were essentially the same.

Pigment extraction and HPLC

Half a gram of fresh leaf tissue was frozen in liquid nitrogen and ground to a powder with a mortar and pestle. The powder was extracted for 8 h with 3 ml acidic methanol (1% HCl in methanol), followed by a second extraction with 6 ml chloroform and 3 ml distilled water. The extracts were vortexed and then centrifuged for 2 min at 3,000g. The spectra were recorded using a SpectraMAX 250 spectrophotometer plate reader (Molecular Devices, Sunnyvale, CA). Extractions for HPLC analysis were done as above, and the resulting supernatant was diluted with 5% acetic acid in a 1:10 ratio and immediately loaded onto the HPLC. High-performance liquid chromatography was done using a Dionex GP40 gradient pump (Dionex, Sunnyvale, CA) using a Microsorb 100-5 C18 column (Varian, Palo Alto, CA). Data were collected and analyzed using PEAKNET software (Dionex). Pigment separation was by gradient elution with a flow rate of 0.75 ml/min and solvent A: 5% acetic acid; solvent B: acetonitrile. The protocol consisted of 1 min at 90% A, 10% B; from 90% A, 10% B to 65% A, 35% B in 17.6 min; to 100% B in 2.4 min, at 100% B for 1 min; to 90% A, 10% B in 3 min; at 90% A, 10% B for 3 min. Absorbance was detected at 380 nm using a Dionex AD20 detector.

Real-time PCR

Real-time PCR primers were designed using the Primer3 software [45]. The primers for the histone deacetylase gene (GenBank AI438666) were: AAGGCTGCTGAACTACAC (forward primer) and TTGACCAGCACACTCAAG (reverse primer); for the cytosine 5' DNA methyltransferase gene (AW215926): CCCGCAAATTCATAGCTG (forward primer) and AGGCCAATCAGTGAAAG (reverse primer); for the methyl-binding protein gene (AI737448): ATGCAGAGCCAAATCAGC (forward primer) and AAGGCAGAGGCACAAAAG (reverse primer); for the *RAD5* gene (AI691852): GCACAACAGCAGCTAAAC (forward primer) and

GCAGCGAATGATTTCTGG (reverse primer); for the cysteine proteinase gene (AW129800): GCTCCCCTTAGCACTATCAC (forward primer) and GACGTGGGTGCTTGTCTT (reverse primer); for the membrane protein Mlo5 (BE025314): AAGACGAACTCTTCGGAGTC (forward primer) and TGCTCTTCTCATCCACTTC (reverse primer), for the snRNP Sm protein F (AW330881): ACGTTGCTGCATTCTTC (forward primer) and TTGGCAAGCTGGAGATTC (reverse primer), for AW433427: ACGGAGAATTAGGGTTCGAT (forward primer) and GTCACTACCTCCCCGTGTC (reverse primer). As an internal control, primers for a thioredoxin-like gene (AW927774) were used: GGACCAGAAGATTGCAGAAG (forward primer) and CAGCATAGACAGGAGCAATG (reverse primer). Fifteen micrograms RNA was used for cDNA synthesis using Superscript II reverse transcriptase (Life Technologies). Real-time PCR was carried out in a reaction containing 1x Mg-free buffer, 2 mM MgCl₂, 200 μM mixed dNTPs, 0.4 μU DyNAzyme II (MJ Research, South San Francisco, CA), 0.5x SYBR Green I (Molecular Probes, Eugene, OR), 0.25 μM of each primer, and 50 ng cDNA in a final volume of 20 μl. Three replicates were performed for each sample plus template-free samples and other negative controls (reaction without reverse transcriptase). Real-time PCR was carried out in a DNA Engine OPTICON2 (MJ Research); the standard amplification protocol consists of an initial denaturation step at 95°C for 30 sec, followed by 40 amplification cycles at 95°C for 10 sec, 58°C for 5 sec, and 72°C for 10 sec. Fluorescence measurements were taken at the end of the annealing phase at a temperature 4°C lower than the melting point of each amplicon. To confirm the size of the real-time PCR products (each less than 200 base pairs (bp)), and that they correspond to a unique and expected PCR product, the PCR products were separated on a 2% agarose gel at the end of the reaction. The PCR products were purified from the gel and sequenced in the Stanford PAN facility to verify their identities. The threshold cycle numbers (Ct) at which each sample reached the threshold fluorescence level for each type of PCR product were determined for all samples. The obtained Ct values for each sample type were used in the formula $2^{(Ct_{ref}-Ct_{tra})}/2^{(Ct_{ref}-Ct_{tra})}$ where Ct_{ref} and Ct_{tra} are the threshold cycles of the reference gene and the gene under study in the UV-B exposed and control samples, respectively. To validate the linearity of the assay, the amplification efficiencies of the target and references were shown to be approximately equal by diluting the template and determining Ct values for the dilution series. Two amplicons have the same efficiency if the differences in Ct values are proportional to the cDNA dilution [46].

Additional data files

The following additional data are included with the online version of this article: a table showing the transcripts regulated by UV-B radiation in *b*, *pl* plants, organized according to their expression profiles with self-organizing maps (Additional data file 1); a table listing the ESTs upregulated by UV-

B in adult leaves covered with PE plastic (Additional data file 2).

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