Analysis of Leaf Proteome after UV-B Irradiation in Maize Lines Differing in Sensitivity*\\n
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UV-B radiation causes diverse morphological and physiological responses in plants, but the underlying mechanisms governing these integrated responses are unknown. In this study, we systematically surveyed responses of maize leaves to UV-B radiation using DIGE 2D gels and identified selected proteins by mass spectrometry and immunodetection analysis. To identify changes in protein accumulation in response to UV-B radiation, a line (b, pl W23) deficient in flavonoid sunscreen compounds and hence similar to commercial corn was used. In addition, its proteome in natural UV-B conditions was compared with that of two maize landraces from high altitudes (Cacahuacintle and Conife Puno). That have improved UV-B tolerance. Protein patterns in adult maize leaves (Zea mays) were documented after growth for 21 days in sunlight depleted of UV-B radiation or growth in sunlight including an 8-h UV-B supplementation during 1 day in the field. We found that there is a very high correlation between previously documented mRNA accumulation assessed by microarray hybridization and quantitative real time reverse transcription-PCR and protein expression after UV-B irradiation in leaves of W23. Multiple isoforms were confirmed for some proteins; at least one protein, pyruvate, phosphate dikinase, is regulated post-translationally by phosphorylation by UV-B exposure. Proteins differentially regulated by UV-B radiation in W23 with higher levels under similar UV-B conditions in high altitude plants were also identified. These could be genetically fixed traits conferring UV-B tolerance and offer clues to specific adaptations to living in high ambient UV-B conditions. Molecular & Cellular Proteomics 4:1673–1685, 2005.

Terrestrial life evolved only after the formation of a stratospheric ozone layer that absorbed most of the damaging UV-B radiation (280–315 nm) in sunlight (1). Recently chloroﬂuorocarbons and other pollutants have catalyzed depletion of this critical ozone shield; consequently terrestrial levels of UV-B radiation are increasing with potentially deleterious consequences for many organisms. Because plants must absorb photons in sunlight to power photosynthesis, they are inevitably exposed to this damaging UV-B radiation (2–4). Adaptations to UV-B radiation include both protection, such as accumulation of UV radiation-absorbing pigments (5–7), and damage repair, such as the use of UV-A photons by photolyases to reverse a subset of UV radiation-induced DNA lesions (8). Because of its absorption spectrum, DNA is a major and long studied target of UV-B damage, but UV-B radiation can also directly damage proteins and lipids; we recently found that UV-B radiation cross-links RNA to particular ribosomal proteins with a concomitant decrease in translation and conclude that RNA is also a major target of UV-B radiation (9).

Plants respond to UV-B radiation through physiological and morphological acclimations (2–4), but the underlying mechanisms governing these integrated responses are unknown. In mammalian cells, UV-B radiation induces the rapid activation of c-fos and c-jun (10, 11); this induction is mediated through several signal transduction pathways (12, 13), including multiple MAP1 kinase pathways. Plant responses to UV-B radiation are also likely to involve multiple signal transduction cascades; thus far, changes in intracellular calcium, calmodulin, serine/threonine kinases, and phosphatase activities have been implicated in UV-B radiation-mediated transcriptional activation of chalcone synthase (14, 15), which catalyzes an initial step in flavonoid sunscreen biosynthesis. UV-B radiation has also been proposed to act through the octadecanoid pathway in tomato (16). In suspension cell cultures of wild tomato Lycopersicon peruvianum, two highly similar MAP kinases, LeMPK1 and LeMPK2, are activated in response to different stresses, including UV-B radiation, whereas an additional MAP kinase, LeMPK3, is only activated by UV-B radiation (17). Therefore, some UV-B signal pathways are shared with other environmental perturbations, whereas additional pathways may account for UV-B radiation-specific responses.

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The initial signal(s) that activate UV-B radiation-specific responses in plants are also undefined. UV-B radiation stimulates production of reactive oxygen species such as singlet oxygen, superoxide radicals, hydroxyl radicals, and H$_2$O$_2$; these compounds may be key regulators of UV radiation-induced signaling pathways (18, 19). Alternatively UV-B radiation may activate receptors directly as exemplified by studies of systemin receptor in L. peruvianum (20). In addition, signaling cascades elicited by the initial products of DNA or other damage can also govern the diverse physiological and developmental responses caused by exposure to this radiation.

By transcriptome profiling we documented diverse physiological acclimation responses in adult maize leaves (Zea mays) grown in sunlight filtered to remove UV-B radiation for 21 days or with ambient UV-B radiation in sunlight plus 8 h UV-B radiation supplementation during 1 day (21). A comparison among near isogenic lines with varying levels of flavonoid sunscreen indicated that the W23 b, pl flavonoid-deficient line showed more differentially regulated transcripts than near isogenic flavonoid-containing lines (21) as expected if sunscreen pigments attenuate UV-B dosage (5). Consistent with prior studies on individual genes, several stress-related pathways were up-regulated by UV-B radiation, whereas genes encoding products required for photosynthesis were down-regulated (21). Additionally dozens of candidate genes and pathways were identified that had not been previously associated with acclimation to UV-B radiation, such as increased expression of ribosomal proteins (21).

A major question stemming from transcriptome profiling is whether the changes in mRNA levels will be mirrored by changes in the corresponding protein abundances or whether physiological changes are effected primarily through post-translational regulation. Two-dimensional (2D) gel electrophoresis is a sensitive and powerful technique for resolving hundreds of proteins in parallel. Combined with mass spectrometry, it allows rapid and reliable protein identification and can provide information about abundance and post-translational modifications. The aim of this study was to systemically survey molecular responses of maize leaves to UV-B radiation using DIGE 2D gels followed by identification of selected proteins using mass spectrometry and Western blot analysis. Given its heightened sensitivity to UV-B radiation and similarity to commercial maize varieties that have been bred to lack red anthocyanin pigments, the b, pl W23 flavonoid-deficient line was used. We found that there is a very high correlation between mRNA and protein expression after UV-B irradiation in leaves of W23 lines. Additionally multiple isoforms of some proteins were confirmed, and for pyruvate, phosphate dikinase (PPDK) we identified a new, reversible phosphorylation induced by UV-B exposure. To gain insight into the basis for tolerance to increased UV-B radiation, we analyzed two maize landraces from high altitudes: one from Mexico (Cacahuacintle, from altitudes between 2200 and 2800 m) and one from the Andes (Confite Punoño, from altitudes between 3600 and 3900 m). Because growing a successful maize crop requires substantial sunlight, these natural habitats receive much higher levels of UV-B radiation than those in lower lands at similar latitudes; such indigenous landraces are predicted to have improved UV-B tolerance. Our recent studies have established that leaves of the five high altitude lines sampled contain more maysin or its precursor, two UV-B radiation-absorbing flavones, whereas these compounds are typically restricted to the silks of maize inbred lines (22). Additionally these five high altitude lines exhibit lower induction of “stress” genes than the low altitude W23 line by microarray hybridization. Therefore, we predicted that high altitude races will have additional as yet undefined adaptive mechanisms of UV resistance and sought to identify proteins differentially regulated by UV-B radiation in W23 and with higher levels under similar UV-B conditions in the high altitude lines. Quantitative differences in protein expression can be genetically fixed traits conferring UV-B tolerance and would thus be specific adaptations to living in a high UV-B environment.

**EXPERIMENTAL PROCEDURES**

**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 Ref. 21). Confite Punoño and Cacahuacintle, two Z. mays highland lines, were obtained from the Germplasm Resources Information Network (GRIN, www.ars-grin.gov/cgi-bin/npgs/html/crop.pl?789).

**Radiation Treatments and Measurements**—Field treatments used plastic filters to screen UV-B radiation (UV-B exclusion) or UV-B lamps (UV-B supplementation, biologically effective UV irradiance of 0.36 W m$^{-2}$ normalized to 300 nm (9 kJ m$^{-2}$ day$^{-1}$; Ref. 23). Solar UV-B radiation was removed to produce the minus UV-B treatment using polyester (PE) filters (100–μm clear polyester plastic; Tap Plastics, Mountain View, CA). This PE filter absorbs UV-B radiation without significantly diminishing UV-A or visible radiation (21). To control for differences in wind or humidity under plastic sheeting, cellulose acetate (CA) sheeting was placed over a control group (100–μm extra clear cellulose acetate plastic, Tap Plastics); the CA sheeting transmits most radiation from sunlight. Seeds were planted outdoors at the Stanford Plant Growth Facility. Approximately 14 days after sowing and 7 days after germination, 1.5 x 3.2 m of each plastic was draped over 1 x 2.5-m wooden frames; the excess plastic was stapled to the sides of the frames to reduce light exposure but permit air circulation; and temperature, humidity, and radiation conditions were measured as described previously (21). There were no significant differences in the growth conditions except manipulation of the UV-B treatment. Measurements of incoming solar radiation were obtained 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 filter was recorded periodically with 1-nm resolution across the entire sunlight spectrum (290–800 nm) to check for changes in the transmittance of the filters. Under our conditions, there were no significant reductions in transmittance after 3 weeks of exposure to solar UV-B radiation, equivalent to the duration of our experiments.

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2 P. Casati and V. Walbot, manuscript in review.
Consequently the filters were not replaced, but as they accumulate dust and could develop small tears, they were cleaned at least every 4th day, and areas with small rips were replaced. Plants were grown under the specified conditions for 21 days, adult leaf samples from adult leaf 9 or 10 were collected for experiments at 4 p.m. in all treatments. The experiment was repeated using different replicate plots (with at least 10 plants of each genotype per plot) to control the field variables that might influence measurements.

For experiments with supplemental UV-B radiation, plants were grown in the field for 28 days under natural sunlight conditions. After that, plants were illuminated by UV-B lamps using fixtures mounted 30 cm above the plants (Phillips, F40UVC 40 W and TL 20 W/12) for 4 and 8 h, and leaf samples were collected immediately after the end of the treatment; this simulates a 4-fold increase in UV-B radiation at 305 nm compared with the Stanford field in August at noon. The bulbs were covered using CA filters to exclude wavelengths lower than 280 nm. As a control, plants were exposed for the same period of time under the same lamps covered with PE (no UV-B treatment).

For experiments with supplemental UV-B radiation in the greenhouse, plants were germinated under supplemental visible lighting to 10% of summer noon radiation without UV-B radiation for 28 days. After that, plants were illuminated by UV-B lamps using fixtures mounted 30 cm above the plants (Phillips, F40UVC 40 W and TL 20 W/12) for 4 and 8 h, and leaf samples were collected immediately after the end of the light treatment; this simulates a 4-fold increase in UV-B radiation at 305 nm compared with the Stanford field in August at noon. The bulbs were covered using CA filters to exclude wavelengths lower than 280 nm. As a control, plants were exposed for the same period of time under the same lamps covered with PE (no UV-B treatment). The output of the UV-B source in all the experiments was recorded using the Optronics spectroradiometer.

**Protein Extraction**—Approximately 200 mg of plant material was ground in liquid nitrogen using a ceramic mortar and pestle, transferred to a 1.5-ml tube containing 0.2 ml of extraction buffer (100 mM Tris-HCl, pH 8.5, 2% SDS, 1% β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 1 mM PMSF, and Complete protease inhibitor mixture (Roche Applied Science)), and then vortexed for 1 min. After this, the extracts were heated for 5 min at 65 °C and centrifuged at 20,000 × g for 20 min at 20 °C. The supernatant was transferred to a new tube, 0.3 ml of ice-cold Tris (pH 8)-saturated phenol was added, and the sample was vortexed for 1 min. Tubes were centrifuged at 20,000 × g for 15 min at 4 °C, and the upper phase was transferred to a new microcentrifuge tube leaving the interface intact. Extraction was repeated twice with a buffer containing 50 mM Tris-HCl, pH 7.5; proteins were precipitated with 5 volumes of cold 0.1 M ammonium acetate in methanol at −20 °C overnight. The samples were collected by centrifugation at 20,000 × g at 4 °C for 20 min, and the pellet was washed with 1.5 ml of cold ammonium acetate/methanol three times. A final wash used 1.5 ml of cold ethanol. The pellet was resuspended in DIGE buffer (30 mM Tris-HCl, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS).

**Protein Labeling with Cy3 and Cy5 Minimal Dyes**—In all experiments, proteins were labeled with ester derivatives of Cy3 and Cy5 after adjusting pH to 8.5 using the supplier’s instructions (Amersham Biosciences). Proteins were labeled at the ratio of 25 μg of protein:20 nmol of Cy3 or Cy5 protein minimal labeling dye (Amersham Biosciences) in dimethylformamide. After vortexing, samples were incubated for at least 2 h on ice. The reaction was quenched by addition of 1 μl of 1 mM lysine and 20 mM dithiothreitol, and 4% (v/v) IPG buffers pH 4–7 or 6–11 was added (Amersham Biosciences).

**Two-dimensional Gel Electrophoresis**—Fifty micrograms of a Cy3-labeled sample was mixed with 50 μg of Cy5-labeled protein prior to 2D gel electrophoresis. An IPGPhor instrument (Amersham Biosciences) was used for isoelectrofocusing with precast IPG strips (pH 4–7, linear gradient, 24 cm; pH 6–11, linear gradient, 18 cm (Amersham Biosciences)). For pH 4–7 IPG strips, 450–μl samples containing 50 μg of protein from each fraction were loaded by in-gel rehydration; whereas for IPG strips with a pH 6–11 gradient, the strips were hydrated in the absence of protein sample, and samples were loaded by cup loading.

The strips were subjected to IEF for 68,000 V·h using the Ettan Dalt system (Amersham Biosciences). Focused gel strips were equilibrated in SDS equilibration buffer (50 mM Tris-Cl, pH 8.8, 30% glycerol, 2% SDS, 6 M urea), first with buffer containing 1% (w/v) DTT for 15 min and afterward with buffer containing 4% iodoacetamide for 15 min. The strips were washed briefly with running buffer, then loaded on top of a prepared SDS-PAGE Laemmli gel cast with 12.5% acrylamide, and covered with 0.5% agarose. Proteins were separated at 3 W per gel for 12–15 h, and the gels were scanned while still between two low fluorescence glass plates at 532 nm (Cy3: excitation, 540 nm, emission peak, 590 nm) and at 633 nm (Cy5: excitation, 620 nm; emission peak, 680 nm) using a Typhoon 8600 fluorescence scanner (Amersham Biosciences). Data were saved in .gel format using ImageQuant software (Amersham Biosciences).

To excise samples for mass spectrometric analysis, a preparative gel loaded with 1 mg of protein was run. Gels were formed between low fluorescence glass plates and bonded to one plate using PlusOne Bind-silane solution (Amersham Biosciences) according to the manufacturer’s guidelines.

**Gel Image Analysis**—Images were analyzed using Phoretix 2D Evolution software (Nonlinear Dynamics Ltd., Newcastle, UK) using the following protocol. Spot detection and background subtraction were performed for the gel image sets imported in gel format image files. Artifacts (such as dust particles or streaks detected as protein spots) were removed by manual editing. When appropriate, spot chains were manually split into separate entities.

This software automatically generated a global reference gel based on the gel containing the most spots, which provides an effective spot index for the analysis of multiple gels. Averaged gels were generated for each of the gel sets containing replicated biological samples. The averaged gels are a statistical combination of several gels to produce a gel that has mean spot values and associated error terms and provides information about spot variation within the gel set. These gel groups were created for spot pattern comparison.

The averaged and reference gels were then recreated to reflect the amended spot detection. Background detection and normalization were performed again to account for any changes in overall volume caused by the manual editing. Once all editing and rematching had been completed, the gels were analyzed for protein spot differences. To compare protein profiles between two samples that had been run on the same gel or between samples run on different gels, a normalization procedure was used to allow for variation in total protein loading onto the gels(s). Total spot volume was calculated, and each spot was assigned a normalized spot volume as a proportion of this total value. Normalized spot volumes were compared between Cy3- and Cy5-labeled samples on each gel. Excluding spots that were unmatched to the reference gel did not affect relative quantification of matched spots to a significant extent. Difference thresholds were then applied to identify the proteins with a statistically significant 1.5-fold difference in normalized spot volume (p ≤ 0.05).

**In-gel Digestion, Mass Spectrometry, and Database Searching**—Before spot picking the gel was stained using SYPRO ruby stain (Amersham Biosciences). The spots were excised using an Ettan Spot Picker (Amersham Biosciences) fitted with a 1.5-mm spot cutter head. Spot picking coordinates were set using Z3 software (Compugen, Tel Aviv, Israel).

The gel spots of interest were excised and subjected to in-gel digestion (donatello.ucsf.edu/ingel.html) with trypsin (porcine, side chain-protected; Promega, Madison, WI). Briefly specific excised...
samples were washed twice with 50% acetonitrile in 25 mM ammonium bicarbonate (NH₄HCO₃) and vacuum-dried. The gel pieces were next reduced with DTT (10 mM in 25 mM NH₄HCO₃, 56 °C for 1 h) and alkylated with iodoacetamide (55 mM in 25 mM NH₄HCO₃, room temperature for 45 min). Then the gel pieces were vacuum-dried, rehydrated in 8 μl of digestion buffer (10 ng μl⁻¹ trypsin in 25 mM NH₄HCO₃), and covered with 20 μl of NH₄HCO₃. After overnight digestion at 37 °C, peptides were extracted twice with a solution containing 50% acetonitrile and 5% formic acid. The supernatants were donated by Dr. Sergio Colombo, spinach Rubisco large subunit was from Dr. Alejandro Viale, and maize PPDK was from Dr. Raymond Chollet. Bound antibody was visualized by linking to alkaline phosphatase-conjugated goat anti-rabbit/mouse IgG according to the manufacturer’s instructions (Sigma). The molecular masses of the polypeptides were estimated from a plot of the log of molecular mass of marker standards (Bio-Rad) versus migration distance.

RESULTS

To investigate the expression of maize proteins regulated during several UV-B irradiation regimes we used a W23 b, pl anthocyanin-deficient line. The B and PI transcription factors strongly induce expression of chalcone synthase, the first enzyme in the flavonoid biosynthetic pathway and subsequent steps leading to anthocyanin pigments (25). W23 plants were grown in the field for 3 weeks under plastics designed to transmit full sunlight UV radiation (CA; solar UV-B radiation) or no UV-B radiation (PE; no UV-B treatment). In addition, a set of plants in sunlight were illuminated for 8 h with an intensity 4-fold higher than solar UV-B radiation (supplementary UV-B radiation). Samples were collected from adult leaves; for each treatment, at least three protein samples were prepared, representing at least three biological replicates of the field experiments. Two maize landraces from high altitudes, Cacahuate and Confluente Punoño, which show higher UV-B tolerance than W23 as described in the Introduction, were used to compare protein patterns with those in W23 under solar UV-B conditions (no plastic, solar UV-B radiation). To compare UV-B responses in a more controlled environment, we tested the effect of supplementary UV-B radiation with an intensity about 4-fold higher than is present in sunlight by giving W23 greenhouse-grown plants a 4- and 8-h treatment. After all treatments, plants looked healthy, independent of the intensity of UV-B radiation they received.

DIGE 2D gels were used to identify spots modulated by UV-B radiation. Table I summarizes the pairs of treatment-genotype combinations compared by DIGE. Following electrophoresis, spots were resolvable in images generated from either Cy3 or Cy5 fluorescence in the labeled proteins. The 2D gels were highly reproducible and had well resolved spots without streaking. An example of reproducibility is shown in Fig. 1 utilizing a dye swap experiment with biological replicate samples from W23 samples that were grown without UV-B radiation (PE filter) and samples from plants that were grown under full solar UV-B radiation (CA filter). For each analysis, isoelectric focusing was performed using two overlapping pH gradients, 4–7 and 6–11. This approach increased the probability of separating proteins with similar isoelectric points and therefore enhances resolution. In both panels virtually all resolved spots are yellow indicating a similar intensity of the Cy3- and Cy5-labeled proteins.

The computer program Phoretix 2D Evolution (Nonlinear Dynamics Ltd.) was used for gel analysis. Triplicate gels were first matched to create an average gel containing those spots observed among all three gels. Only spots that met several
 stringent criteria were analyzed further (see “Experimental Procedures”). The analysis of the two overlapping pH gradients identified 178 protein spots with differential intensities of 1.5-fold in the various comparisons using the W23 genotype (Table I).

When comparisons were performed between W23 and the two high altitude lines, many differences in shifted spots were observed (see Supplemental Fig. 1). Spot shifting in many cases likely reflects charge or size polymorphisms in the protein sequences, which in turn reflect the ~1% average divergence in gene sequences between the different genotypes of maize (26). To identify candidate proteins that might contribute to UV-B tolerance in the high altitude lines, we looked for proteins present in both W23 and the high altitude lines that showed higher constitutive expression in the landraces than in the non-adapted W23 line. From these we selected those proteins that also showed a change in intensity after any of the UV-B treatments in W23. Fifty-three protein spots met these criteria. These proteins are likely to be genetically fixed traits conferring UV-B tolerance in the landraces, that is, they are specific adaptations to living at high altitude.

Protein Identification and Expression Patterns after UV-B Exposure—Spots that were up- or down-regulated 1.5-fold as measured by DIGE were subjected to protein identification by mass spectrometry analysis as described under “Experimental Procedures.” Interpretable MS/MS spectra were obtained for 163 of the 178 spots that were differentially expressed across the overlapping pH ranges. Protein identifications are shown in Supplemental Table 1. For some spots, peptide sequences for more than one protein were obtained. In these cases, spot identities were assigned based on the fit of the theoretical pI and molecular weight of each protein to that experimentally derived from the 2D gels. If an ambiguity arose, the spots corresponding to the same protein from different gels were reanalyzed by mass spectrometry for clarification of the identification.

To identify coordinately regulated proteins in the W23 line a hierarchical clustering method was applied. We found two main groups (Fig. 2, A–D) corresponding to proteins that are increased by at least one of the UV-B treatments (Fig. 2, A and C) or decreased under the same conditions (Fig. 2, B and D). Consistent with microarray data (9, 21), more proteins showed increased than decreased levels after UV-B treatments. As shown in Fig. 2, most of the selected proteins respond to

### TABLE I

| Experimental conditions | Sample 1               | Sample 2               |
|------------------------|------------------------|------------------------|
| Field-UV-B treatment 1 | W23-no UV-B            | W23-solar UV-B         |
| Field-UV-B treatment 2 | W23-solar UV-B         | W23-supplementary UV-B |
| Greenhouse-4-h UV-B    | W23-no UV-B            | W23-supplementary UV-B |
| Greenhouse-8-h UV-B    | W23-no UV-B            | W23-supplementary UV-B |
| Field-solar UV-B       | W23                    | Confite Puneño          |
| Field-solar UV-B       | W23                    | Cacahuacintle          |

* The no UV-B treatment was achieved using a PE plastic screening; solar UV-B treatments included a CA plastic covering that transmits UV-B radiation; UV-B lamps were used to increase UV-B irradiation in the supplementary UV-B treatment.
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A

W23

Solar UV-B+ 4h+UV-B

Confession/W23

B

C

D

Solar UV-B+ 4h+UV-B

Confession/W23

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UV-B radiation in natural sunlight conditions, and a subset is further changed by UV-B supplementation in the field. Sixty-eight of the 178 proteins (spots) are changed by supplementary UV-B radiation in greenhouse conditions where the plants receive no UV-B radiation prior to the treatment period; only 37 proteins are regulated by both field and controlled greenhouse conditions (Fig. 2). Collectively these results confirm that deviation from ambient conditions in sunlight is a significant variable controlling gene expression in plants rather than simply a response to UV-B dosage per se. This proteomic analysis also confirms the general conclusions from previous transcriptome studies after the same UV-B treatments of adult leaves of the W23 b, pl genotype (21).

Interestingly 19 proteins had increased levels after some UV-B treatment regimes but decreased levels in other UV-B treatments. Two examples include a putative ribosomal protein S5 (Fig. 2A) and a 33-kDa oxygen-evolving protein (Fig. 2C). These proteins may be subject to different pathways of UV-B regulation that respond to specific dosages or qualities of light conditions. In total, 53 proteins that showed increased levels in at least one of the treatments given to W23 also showed increased levels in high altitude plants compared with W23 in the full sunlight UV-B condition. These proteins belong to diverse functional groups, and therefore no single pathway was identified as responsible for the enhanced UV-B tolerance of the high altitude lines.

**UV-B Radiation-regulated Proteins**—The central aim of this work was to identify proteins quantitatively affected by UV-B radiation, and one of the specific goals was to compare the proteomic results with previously published microarray results obtained from samples collected after the same UV-B treatments in the same maize lines (9, 21).3 Additionally the proteomic analysis can discover translational regulation and post-translational modifications that are missed by microarray hybridization. Fig. 3 summarizes the key findings by the plant processes responding to UV-B treatments based on identification of representative proteins. As already observed in our microarray studies (9, 21), the functional group with the largest number of proteins modulated by UV-B radiation are involved in protein biosynthesis (36 proteins, Fig. 3). Examples include ribosomal proteins, initiation and elongation factors, and ribosome recycling factors; 28 of the identified proteins are increased by UV-B radiation in at least one of the UV-B treatments applied. UV-B radiation can damage ribosomes by forming cross-links between RNA and specific ribosomal proteins; cellular recovery is accompanied by selective translation of ribosomal protein transcripts and increased expression of transcripts for ribosomal proteins and translation factors (27). There are eight proteins associated with protein synthesis that in some conditions are decreased by UV-B treatments; it is possible that these factors are regulated by translational or post-translational processes rather than by a change in protein level.

To ascertain whether the observed changes in gene expression translate into similar changes in protein expression, we carried out a comparison of mRNA and protein expression. Eighty-six of the 163 identified proteins were found to be UV-B radiation-regulated both by transcriptome studies and by this proteomic study (Supplemental Table 1). Among these, only seven showed a different pattern of UV-B regulation in the two methods of analysis. An important difference in precision between the two assays is that we used cDNA microarrays, which can detect different members of a gene family, whereas in this proteomic study individual gene products were resolved into distinct spots. Most of the proteins whose changes were not detected by microarrays belong to the same functional groups highlighted by the transcriptome profiling experiments. It is important to mention that the microarray slides previously used included a subset of the genes that can be expressed in adult leaves under the conditions of study (a total of 8,000 cDNAs in two different array sets); 25 of the genes encoding proteins regulated by UV-B radiation in this study were not included in the arrays.

**Western Blot Validation of 2D DIGE Results**—Immunoblot experiments were performed to assess the interpretation of results from the 2D gels, comparing leaf proteins of plants under several UV conditions. Fig. 4A shows examples of the results using antibodies against β-actin, histone H4, and ADP-glucose pyrophosphorylase; for each of these proteins, only one protein spot was identified after 2D gel analysis. There is a good correlation of the results obtained by the two techniques. For β-actin, both methods clearly show that there is an increase of protein after solar UV-B irradiation compared with the no UV-B treatment under field conditions, and β-actin is also higher in the high altitude lines than in W23. However, treatment in the greenhouse does not change protein levels.

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3 P. Casati and V. Walbot, unpublished data.
For histone H4, there is a decrease in the protein levels after both UV-B treatments in the field; whereas for ADP-glucose pyrophosphorylase, there is an increase in enzyme levels by the same conditions. Thus, at least for this set of proteins with only one form changed by UV-B radiation in the 2D gels, the immunoblot analysis validates the results.

On the other hand, from Supplemental Table 1, it is clear that some proteins are represented by more than one spot. These spots may represent different products of two very closely related genes or post-translational modifications of a single protein. Table II lists the subset of these proteins that showed opposite regulation by UV-B radiation. To investigate these differences further, immunoblot analysis was done using antibodies against some of these proteins (Fig. 4B). As shown in Fig. 4B, Rubisco large subunit, phosphoenolpyruvate carboxylase, and pyruvate, phosphate dikinase exhibit similar total levels in all treatments as determined by immunoblot detection. This means that there are probably different isoforms regulated in opposite ways or that one isoform is post-translationally regulated. Unfortunately these possibilities cannot be distinguished by immunoblot blots after one-dimensional gels. In contrast, for two heat shock proteins, differences in total levels of proteins by Western blots are observed. For HSP70 (Fig. 4), there is a decrease in protein levels after UV-B irradiation in field conditions (both by solar and supplementary UV-B radiation). Four spots corresponding to this protein were identified by MS/MS after recovery from the 2D gels; three of these show the same down-regulation by UV-B in field conditions (Fig. 2 and Table II), whereas only one was up-regulated. Thus, down-regulation of three protein spots can account for the observation with Western blots. For UV-B treatments in the greenhouse, there is an overall increase of protein levels measured by immunoblot. Only two of the four spots in 2D gels are regulated by UV-B radiation in the field (Fig. 2 and Supplemental Table 1), and these two spots are regulated in opposite ways. In contrast, there is an increase observed by immunoblots in greenhouse conditions. Of the three spots identified by MS/MS, two are increased by UV-B radiation in the greenhouse, and only one is decreased, and this can also explain the overall increase observed by immunodetection.

**PPDK Is Post-translationally Modified**—For the proteins listed in Table II, which showed multiple spots with opposite regulation, we investigated whether post-translational regulation could account for these differences. Thus, possible phosphorylated, acetylated, methylated, and carbamylated peptides where queried in the mass spectra results for the selected spots. PPDK was identified four times (Table II) and is resolved as a string of discrete spots of 102 kDa, pl ~5 (Fig. 1). This enzyme plays a crucial role in generating carbon skeletons for carbon fixation in maize. A novel phosphorylation site was identified at Thr-526 (spot number 71 in Supplemental Table 1) for PPDK as shown in the CID MS/MS spectra (Fig. 5). This spot is decreased by solar UV-B radiation and further decreased by supplementary UV-B radiation in the field (Supplemental Table 1), but it is increased by UV-B radiation in greenhouse conditions. For other PPDK spots (spot numbers 9 and 10 in Supplemental Table 1), no MS/MS data for the phosphorylated peptides were obtained for further confirmation. These two spots show opposite UV-B regulation to phosphorylated spot 71. Consequently there is clear evidence for regulation of the phosphorylation state of Thr-526 by UV-B radiation (Fig. 1). Because the same spot is increased in greenhouse experiments, the mechanisms of regulation are complicated, and post-translational status may respond to differences in light qualities. Phosphorylation of PPDK has been described previously in maize (28). PPDK activity in the leaves of C4 photosynthesis plants such as maize is strictly regulated by light; the activity decreases in darkness and recovers rapidly upon illumination. Such responses are regulated by phosphorylation and dephosphorylation of PPDK to modulate carbon fixation; this regulation is mediated by a different pyruvate, phosphate dikinase regula-
tory threonine residue (Thr-456 in maize) in the enzyme (28). Thus, a different Thr residue appears to regulate the activity of this enzyme by UV-B radiation.

For most of the other proteins, covalent modifications were not readily identifiable. Because these modifications can be in very low stoichiometry (phosphorylation is generally less than 10%) and some modifications (such as phosphorylation) are labile in the MS analysis, modification of the proteins cannot be ruled out.

**DISCUSSION**

The identification of genes and proteins that are differentially expressed after UV-B irradiation in maize is an important step in elucidating the mechanisms that participate in these responses. To this end, we carried out a proteomic analysis that parallels previous microarray studies by utilizing samples from the same maize lines and radiation conditions (9, 21). We found a very high correlation between mRNA and protein expression for more than half of the proteins identified in this study. The discrepancies observed likely reflect two sources. First, there are diverse modes of regulation of the differentially expressed proteins that do not require a change in mRNA abundance. Second, the microarrays included only about 15% of maize genes. Consequently many of the proteins demonstrated to be differentially regulated by UV-B radiation in this study did not have a representative cDNA in the arrays.

Examples of proteins that may be regulated post-transcriptionally include those for which levels are changed in an opposite direction in the microarray and proteomic studies or for which changes are detected by only one of the two methods. Only eight proteins showed an opposite differential expression in this study and in the previous microarray studies (Supplemental Table 1). There are three different forms of Rubisco large subunit that showed increased levels by UV-B radiation, whereas two other isoforms are decreased (Table II), and the decrease is in agreement with the previous microarray studies (21). Rubisco large subunit is encoded in the chloroplast and is a unique gene (29); however, it is subject to diverse co- and post-translational modifications, including the acetylation of Pro-3, N-formylation, trimethylation in the ε-amino of Lys-14, and carbamylation in the active site; several amino acid modifications occur in some species but not others (30). Thus, even if Rubisco large subunit transcription is down-regulated by UV-B radiation, translational and post-translational regulation probably occurs to the existing protein. For another protein in the group with opposite differential

![Immunoblot analysis of proteins regulated by UV-B radiation.](image)

**TABLE II**

| Protein                              | Total no. of spots | Increased | Decreased |
|--------------------------------------|--------------------|-----------|-----------|
| Phosphoenolpyruvate carboxylase       | 7                  | 6         | 1         |
| HSP70                                 | 4                  | 1         | 3         |
| HSP90                                 | 3                  | 1         | 2         |
| PPDK                                  | 4                  | 3         | 1         |
| ATP synthase α chain                  | 4                  | 2         | 3         |
| ATP synthase γ chain                  | 2                  | 1         | 1         |
| Rubisco large subunit                 | 5                  | 3         | 2         |
| Cytosolic α-phosphoglycerate kinase   | 2                  | 1         | 1         |
| Ferredoxin                            | 3                  | 1         | 2         |
| Chloroplastic aldolase                | 3                  | 2         | 1         |
| Nascent polypeptide-associated protein| 2                  | 1         | 1         |
| Peptidylprolyl isomerase              | 2                  | 1         | 1         |
| Ribosomal protein S5                  | 2                  | 1         | 1         |
| Ribosomal protein L6                  | 2                  | 1         | 1         |

*One spot is increased by one UV-B treatment but decreased by another.*

![Proteomic Analysis of Maize Lines after UV-B Irradiation](image)
regulation at the RNA transcript and protein levels, a putative nascent polypeptide-associated complex α chain, two protein spots were detected where one spot increased and the other spot decreased after exposure to UV-B radiation. It is possible that an unknown post-translational regulation occurs in this protein.

Some proteins were found in more than one spot by MS analysis. This can occur because more than one member of a gene family is regulated by UV-B radiation, or as just discussed, there is post-translational modification of a single gene product upon UV-B exposure. A subset of 14 proteins represented with more than one spot showed opposite regulation by UV-B radiation. For most of the proteins, we could not find any phosphorylated, acetylated, methylated, or carboxylated peptides when we searched the MS/MS results for the selected spots. The only protein with a proven phosphorylation difference after UV-B irradiation was PPDK. A novel phosphorylation site at Thr-526 was detected mainly when solar UV-B radiation was removed with the PE filter in the field. The same spot was also detected in greenhouse experiments when UV-B radiation was supplemented. The complex responses suggest regulation that integrates differences in both light qualities and quantities as often occurs in enzymes participating in photosynthesis. Phosphoenolpyruvate carboxylase is another well studied enzyme involved in CO₂ fixation in the mesophyll cells of corn and other C₄ plants. Its activity is also reported to be highly regulated by light through reversible phosphorylation (31). Similar to PPDK, phosphoenolpyruvate carboxylase was identified multiple times in our study as a string of discrete spots with some isoforms regulated in opposite ways by UV-B radiation (Table II). However, despite its known phosphorylation regulation, we did not detect any phosphorylated peptides.

Maize races grow in diverse environments and exhibit immense allelic and phenotypic diversity. Improved maize germplasm able to tolerate specific stress conditions, such as increased levels of UV-B radiation, is crucial if the productivity of maize-based farming systems is to be sustained or increased. To exploit this diversity in adaptation to high UV-B radiation, we used two maize landraces from high altitudes: Cacahuacintle and Confito Punoño. Both indigenous landraces constitutively contain UV-B radiation-absorbing flavones in their leaves (22) and show far fewer transcriptome changes after UV-B treatments than the W23 low altitude inbred line. Our interpretation is that the high altitude lines have greater shielding against UV-B damage and another as yet undefined adaptive mechanisms of UV resistance. To begin to identify these mechanisms we looked for protein spots present both in W23 and the high altitude lines that showed higher constitutive expression in the well adapted landraces. Fifty-three proteins that showed increased levels by UV-B radiation in at least one of the treatments also showed increased levels in high altitude plants compared with W23. Sixteen showed decreased levels in high altitude plants and were also decreased by UV-B radiation in W23. The abundances of these 69 proteins appear to be genetically fixed traits associated with UV-B tolerance and merit further investigation.

This study identified a number of protein changes associated with UV-B responses in maize. UV-B radiation-regulated
protein expression changes were quantified using DIGE. This method provides significantly more accurate measurements and smaller variation in protein abundance measurement than other 2D gel techniques, such as silver staining (32). Although the limit of detection of 0.25 ng of protein in a spot is similar with silver staining and fluorescence labeling, a major advantage of fluorescent dyes is that their concentration quantification is linear over a 4-order of magnitude range, whereas silver staining has an effective range of only 1 order of magnitude. Because typical tissues have proteins that range from low to very high abundance, the wider range is a great benefit for quantification purposes. DIGE is a particularly powerful approach to study changes in protein levels in studies involving comparison of multiple samples as only one gel is run to compare two samples. Variation in protein spots as a result of gel-specific characteristics impacts both samples within a single gel; therefore, the relative amount of a protein in one sample compared with another is unaffected. In traditional 2D electrophoresis, individual samples are separated in different gels, and then the gels are overlapped to locate modified spots. Often discrepancies between gels in resolution and/or spot intensities make the overlaying of images, correct matching of proteins, and data interpretation very difficult. Another method that is popular with cells cultured in liquid media is incorporation of stable isotopes (33). This method is not feasible for field-grown maize and would be difficult to establish under greenhouse conditions for adult plants; as we report here, there are differences in plant response to UV-B lamps in the greenhouse compared with solar radiation, hence conducting the entire experimental protocol in the greenhouse or growth chamber would yield different results than using field conditions.

Considering all of these factors, DIGE is the method of choice for plant proteomics using materials grown under natural conditions. Although investment in DIGE equipment and supplies is significant, the cost and effort of each experiment are considerably reduced compared with any single label method as only one gel is run per comparison instead of two. Furthermore reliably pinpointing shifted spots and spots of altered abundances can be automated with appropriate software. Presently using a single gel and a broad pH IEF step, ~1,000 spots can be detected. In our work, two different IEF strips with distinct pH ranges (4–7 and 6–11) increased the number of spots that can be detected. Despite this, a large fraction of the genes with altered mRNA levels were not detected in 2D gel analysis of proteins. This is not surprising because gel-based proteome approaches detect proteins that are present in relatively high abundance, whereas transcriptome microarray analysis can detect alterations in mRNAs expressed at very low levels (as an example, see Ref. 34).

Among the proteins shown to display altered expression by UV-B exposure, 13 functional groups were identified including unknown function (Fig. 3). Representation among these groups is similar to those described before by microarray analysis (9, 21). This is not surprising because there is a large overlap in the gene types identified by the two studies. Some of the identified genes are likely to have an important role in UV-B responses as their transcript levels are highly increased after UV-B treatments, and the corresponding proteins are highly expressed and detectable by this proteomic method. There are also a group of proteins that have not been described as regulated by UV-B radiation in the microarray studies, including a guanine nucleotide-binding protein β subunit-like protein (GPB-LR); a translationally controlled tumor protein (TCTP); a DNA-binding protein, GBP16; a putative ATP/GTP-binding protein; and yucca leaf protein (YLP). GPB-LR is the product of the auxin-responsive gene designated arcA (35), but it has been implicated previously as a mediator in the signal transduction pathway during agonist cell cycle arrest by salicylic acid and also by UV-B irradiation (36). GBP16 is a member of a DNA-binding protein complex that specifically binds the single-stranded G-rich telomere sequence (rice G-rich telomere binding protein) (37).

The translationally controlled tumor protein is a guanine nucleotide dissociation inhibitor acting on the translation elongation factor eEF1A (38) and in this role may be important in modulating protein synthesis after UV-B damage. The expression levels of the TCTP are strongly down-regulated at both the mRNA and protein levels during tumor reversion/suppression in animals. eEF1A is a GTPase, and TCTP preferentially stabilizes the GDP form of eEF1A and furthermore impairs the GDP exchange reaction promoted by eEF1B. Thus TCTP has guanine nucleotide dissociation inhibitor activity, and moreover, TCTP is implicated in the elongation step of protein synthesis (38). The increase in the levels of the YLP by UV-B radiation is also noteworthy. YLP is an inhibitor of tobacco mosaic virus. It exhibits potent activity against other viruses in vitro by inhibiting viral multiplication and also by virtue of viricidal activity at higher concentrations (39). YLP strongly inhibits protein synthesis in virus-infected cells but not in uninfected cells (39). Increased levels of YLP and TCTP during UV-B responses may explain, in part, the significant reduction in protein synthesis detectable within 2 h of treatment (27). We found previously that this decrease in protein synthesis by UV-B radiation correlates with ribosomal damage (27) and the increased transcription and translation of ribosomal proteins needed for ribosome replacement (9, 21, 27). YLP and TCTP may act directly to decrease protein synthetic capacity or permit selective translation in the functional ribosomes. Thus, the combined results from all these experiments on cellular damage, transcriptome profiling, and proteome changes are complimentary approaches to understanding modulation of a key cellular process such as translation.

In conclusion, by using DIGE 2D gels followed by identification of proteins by mass spectrometry and Western blot validation we identified a set of proteins in maize leaves that
are changed after UV-B radiation. Altered abundances can reflect both translational and/or post-translational controls. A hundred and seventy-eight proteins were found to be changed in at least one of the treatments applied; some proteins were found in multiple spots, and at least one protein (PPDK) is regulated post-translationally by reversible phosphorylation conditioned after UV-B exposure. Proteins differentially regulated by UV-B radiation in W23 and with higher constitutive levels under similar UV-B conditions in high altitude plants are candidate adaptive traits conferring UV-B tolerance.

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