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Transcriptomic, proteomic and metabolomic analysis of maize responses to UV-B

Comparison of greenhouse and field growth conditions

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UV-B radiation from normal solar fluence elicits physiological and developmental changes in plants under fluctuating environmental conditions. Most UV photobiology studies in plants utilize controlled greenhouse and growth chamber environments in which few conditions vary except the brief presence of UV-B radiation. Our purpose was to compare responses to UV-B in irradiated and shielded maize organs in field (natural solar plus 2x solar supplementation for defined periods) and greenhouse (2x solar supplementation only) conditions during a 4 h exposure. Three parameters were assessed—transcripts, proteins and metabolites—to determine the degree of overlap in maize responses in field and greenhouse conditions. We assessed irradiated leaves, and both shielded leaves and immature ears. After comparing transcriptome, proteome and metabolome profiles, we find there are more differences than similarities between field and greenhouse responses.

Introduction

Sessile plants experience fluctuating environmental conditions, and must therefore acclimate to abiotic conditions that limit their physiological or developmental potential. For example, as a result of ozone depletion, there are periodic spikes in solar UV-B fluence in both temperate and polar regions; even without additional damage to the ozone shield, this protective atmospheric layer is not expected to stabilize until ~2,050.[1] Because plants absorb light for photosynthesis, some organs are also continuously exposed to solar ultraviolet-B radiation (UV-B 290–320 nm). This energetic radiation causes direct damage to DNA, proteins, lipids and RNA in irradiated organs. DNA damage and its repair are the best-understood local consequences of UV-B exposure.[2] Absorption of photons by DNA induces the formation of covalent bonds between adjacent pyrimidines, generating cyclobutane pyrimidine dimers (CPDs) and, to a lesser extent, pyrimidine (6-4) pyrimidone photoproducts (6-4PPs).[3] These lesions disrupt base pairing and block DNA replication and transcription if photoproducts persist, or result in mutations if photoproducts are bypassed by error-prone DNA polymerases.[2] Therefore, plants have evolved mechanisms such as reflective waxes and pigments that reduce UV-B penetration to protect against DNA damage,[4,5] and have also evolved several DNA repair systems to remove or increase tolerance of DNA lesions.[6-8]

On the other hand, by unknown mechanisms UV-B perception (or damage) is transduced into both local physiological changes and developmental alterations in shielded meristems and immature organs.[2,9,10] Using a protocol of irradiating canopy leaves in greenhouse grown maize plants and measuring transcriptome, proteome and metabolite changes in both exposed and shielded organs, we have sought to identify signals that coordinate systemic responses.[11] We found that exposure of just the top leaf substantially alters the transcriptome of both irradiated and shielded organs, with greater changes as additional leaves are irradiated. There is specificity in the responses; for example, some phenylpropanoid pathway genes were expressed only in irradiated leaves and, correspondingly, some phenylpropanoid precursors to sunscreen compounds only accumulated in these leaves.[11] Candidates in early steps of signal transduction and possible signal molecules were also identified in the controlled greenhouse conditions in which no UV-B is present until a singular treatment period. Because field-grown maize experiences fluctuating UV-B levels and variation in other environmental conditions, we have now compared the transcriptome, proteome and metabolome changes after 4 h of supplementary UV-B irradiation in naturally UV-B-acclimated field plants to the same genotype grown in the greenhouse in the absence of UV-B.

Results

Microarray hybridization design, replication and statistical analysis. W23 inbred maize was grown in an irrigated summer field under natural UV-B or under summer greenhouse conditions in the absence of UV-B, and then irradiated for 4 h

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Table 1. Total probes scored as significant hybridization

Sample type	Irradiated leaf (IR)	Shielded leaf (SH)	Immature ear (IE)
Fully irradiated greenhouse (WPI)	32,500	Not applicable	31,500
No irradiation greenhouse (NI)	Not applicable	33,000	34,000
2-leaf irradiated greenhouse (2L IR greenhouse)	28,000	26,000	31,200
2-leaf irradiated field (2L IR field)	32,000	29,000	31,500

in August 2008 with UV-B equivalent to ~two-fold the typical noon summer solar fluence at Stanford University as described in Materials and Methods. To generate irradiated leaf samples, the topmost two leaves were threaded through a slit in polyester (PE) plastic that absorbs UV-B; consequently, the upper canopy leaves received UV-B directly while the next two leaves under the plastic and immature ears tightly encased in husk leaves were shielded from direct irradiation during the explicit UV-B treatment. As controls, we also collected leaves from greenhouse plants that were fully irradiated (whole plant irradiated, WPI) and from control greenhouse plants (non-irradiated, NI) grown in the absence of UV-B.

Transcript analysis was done using a highly sensitive, custom-designed Agilent® 4 x 44K array that contained 60-mer probes and internal spike-in control probes to quantify transcript abundance and non-specific hybridization for ~39,000 maize genes. Hybridization signals were scored as present if the signal was three-fold above the standard deviation of the average hybridization to the negative control (non-hybridizing) probes.[12] Transcriptome differences were assessed from leaf or ear samples pooled from four individuals and four independent biological replicates were performed with symmetrical dye labeling to minimize systematic errors.[13]

As shown in **Table 1**, maize leaves (L) and immature ears (IE) express a substantial number of genes. Under greenhouse conditions, leaves from fully UV-B-irradiated plants (WPI) express 32,500 different transcripts (**Table 1**) and leaves from non-irradiated plants (NI) show a slightly higher number of transcripts expressed (33,000). A similar trend is observed from immature ears from the same plants (**Table 1**). Two leaf exposure decreases transcriptome diversity substantially, both in irradiated (IR) and shielded organs (SH, **Table 1**) in greenhouse-grown plants. In contrast, irradiated leaves of field plants retain 4,000 more transcript types compared to the irradiated leaves in greenhouse-grown plants; ~3,000 transcript types were retained in shielded leaves of acclimated field maize compared to greenhouse plants. In immature ears, transcript number diversity is similar in both greenhouse and field plants (**Table 1**) after the UV-B treatment.

To further assess the transcriptome responses after UV-B treatment, samples from field and greenhouse (2L IR field or greenhouse) were compared to leaves of non-irradiated control plants (NI) from the greenhouse. Irradiated greenhouse plants only received UV-B in the two upper leaves. The acclimated field leaves show 4,667 transcriptome differences after supplementary UV-B treatment (2-fold, $p < 0.05$) compared to the leaves that have never seen UV-B. In contrast, the greenhouse grown plants show fewer changes (699 total transcripts, two-fold, $p < 0.05$) (**Fig. 1A**). Sixty percent of the UV-B-regulated transcripts in greenhouse plants showed a similar UV-B regulation in plants grown in the field. These transcripts are, therefore, associated with responses to a UV-B challenge. We propose that the large number of transcripts unique to field plants compared to the NI controls represent acclimation to UV-B and myriad other environmental variables in field-grown plants.

Common UV-B upregulated transcripts under both conditions include genes in secondary metabolism, in particular genes in the flavonoid pathway; cell wall metabolism; DNA metabolism, including transcription factors, chromatin proteins and DNA repair enzymes; proteins that participate in stress responses; and protein synthesis and degradation, among others (**Fig. 1C and Sup. Table 1**). Genes in these categories have already been demonstrated to be regulated by UV-B in maize,[14,15] and it is now clear that this regulation occurs both under greenhouse and field conditions in the same W23 inbred line. Additionally, UV-B results in decreased transcripts for photosynthetic proteins, chaperones and DNA and RNA binding proteins in both growth conditions (**Fig. 1D and Sup. Table 1**); the particular transcript types have already been identified as downregulated by UV-B in greenhouse conditions[11] and are now confirmed under field conditions.

Next we analyzed the transcriptome changes that occurred in 2-leaf UV-B-irradiated plants grown in the greenhouse (2L IR greenhouse) or in the field (2L IR field) and compared them to the transcriptome of fully UV-B-irradiated plants in the greenhouse (WPI). As we previously reported, only a subset of genes showed differential expression between 2-leaf irradiated plants and fully irradiated plants in the greenhouse (180 transcripts, **Fig. 1B**, 2-fold changes, $p < 0.05$). Differences were substantial, however, when comparisons were done with field samples (**Fig. 1B and Sup. Table 2**), because 4,373 transcripts were expressed differentially between UV-B-irradiated field leaves and leaves from fully irradiated greenhouse plants. Thus, similar responses are observed in greenhouse plants for both two-leaf and whole plant irradiation, but very different and more extensive transcriptome changes occur in field plants acclimated to UV-B when two leaves receive supplementary radiation.

We then compared the transcriptomes from irradiated leaves from the two growth conditions to the shielded leaves. As expected, there is clearly a stronger difference in transcript changes in IR leaves (3,957 transcripts, 2-fold change, $p < 0.05$; **Fig. 2A**) than in SH leaves (605 transcripts, 2-fold change, $p < 0.05$; **Fig. 2A**). One hundred and ninety-two transcripts are changed similarly both in IR and SH (intersection of Venn diagrams, **Fig. 2A**), representing less than 5% of the total differences between greenhouse and field plants. Moreover, in comparing shielded to irradiated leaves within a sample type (greenhouse or field plants), there are 3,060 significant differences in the greenhouse sample approximately equally divided between up and downregulated classes, but only 387 significant changes in the field comparison, with roughly 70% being upregulated (**Fig. 2B**). This result highlights the sensitivity of greenhouse plants to a single irradiation

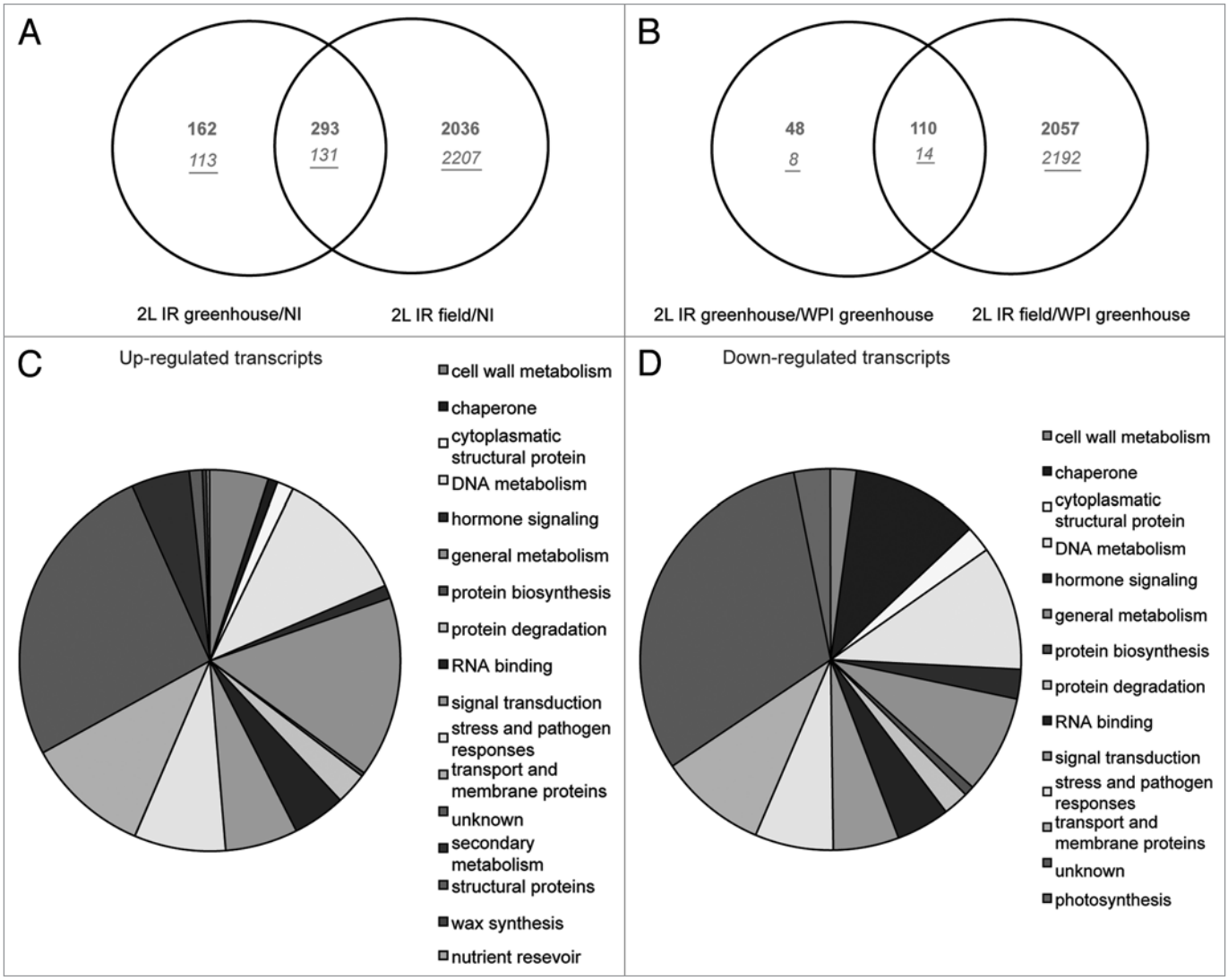


Figure 1. Transcriptome changes comparing two irradiated leaves to leaves from non-irradiated (NI) and fully UV-B irradiated (WPI) greenhouse plants. Upregulated genes are red in bold, downregulated genes are green, underlined in italics. (A) Intersection of genes differentially expressed in two irradiated greenhouse leaves (2L IR greenhouse) and field plants with two irradiated leaves receiving supplementary UV-B (2L IR field), compared to leaves from non-irradiated plants (NI). (B) Intersection of genes differentially expressed in irradiated greenhouse leaves (2L IR greenhouse) and field plants with two irradiated leaves receiving supplementary UV-B (2L IR field), compared to leaves from fully UV-B irradiated greenhouse (WPI) plants. Transcripts changed by 2-fold ($p < 0.05$) are included. (C and D) GO classification of common UV-B-regulated transcripts in irradiated leaves from field and greenhouse plants compared to non-irradiated leaves based on their putative function: (C) upregulated and (D) downregulated.

treatment compared to the field-grown plants which have experienced 5 wks of natural solar radiation. Furthermore, there are only 165 genes regulated in a parallel manner (127 up and 38 downregulated) in the two sample types; this constitutes ~5% of the transcriptome changes in the greenhouse plants and 43% of the responses in the field leaves (center element, **Fig. 2B**). Of the 127 commonly induced transcript types are those encoding enzymes in the phenylpropanoid pathway, participating both in flavonoid synthesis (chalcone synthase, flavonoid-3,5-hydroxylase and a UDP-glucose glucosyltransferase) and cell wall metabolism (cinnamic acid 4-hydroxylase and the phenylalanine ammonia-lyase). These data indicate that a single, moderate dose of UV-B similar to a UV-B spike in natural environments elicits responses that should result in higher sunscreen levels, even in leaves that have been exposed daily to solar UV-B. A full gene list is found in **Supplemental Table 1**.

Additionally, transcription factors (MADS box, MYB and WRKY family proteins, a *Glossy1* homolog) and signal transduction proteins (a calcium:calmodulin-dependent protein kinase CaMK3 and a Ras-related protein ARA-4) were upregulated by UV-B in both field and greenhouse conditions in this comparison of irradiated to shielded leaves within the same treatment. Therefore, although field and greenhouse plants differ dramatically in the scope of transcriptome changes, there are some common responses such as flavonoid synthesis, changes in the cell wall biosynthetic genes and activation of certain pathways by transcription factors and signal transduction pathways. Of the common downregulated transcripts, there are also several

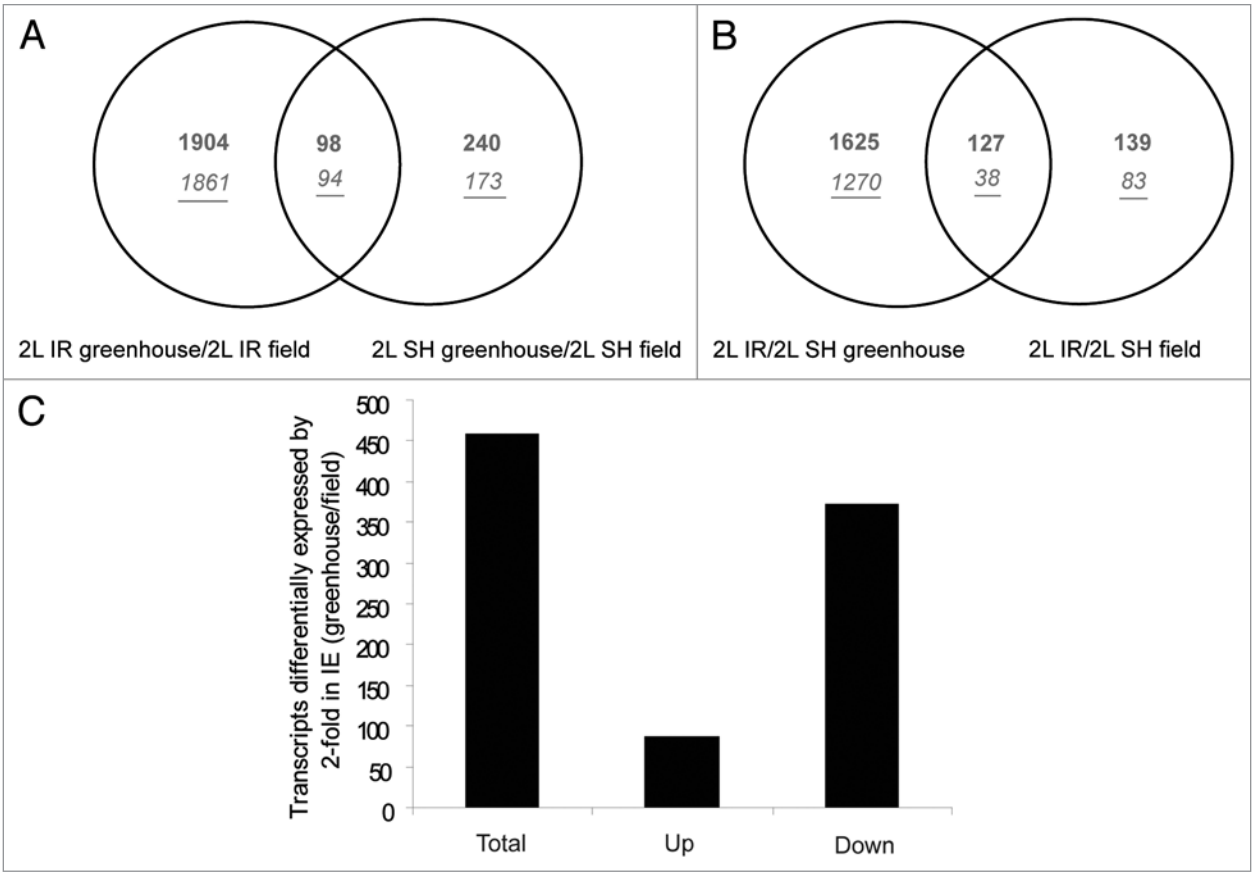


Figure 2. Transcriptome changes comparing irradiated leaves, shielded leaves and immature ears from field and greenhouse plants. (A) Transcripts that show differential expression in irradiated or shielded leaves in the greenhouse compared to similar leaves from field plants. (B) Transcripts that are changed in irradiated (IR) in comparison to shielded (SH) leaves in the greenhouse or field samples. Upregulated genes are red in bold; downregulated genes are green, underlined in italics. (C) Transcripts differentially expressed by two-fold in immature ears (IE) from 2-leaf UV-B-irradiated greenhouse versus field plants. Transcripts changed by 2-fold ($p < 0.05$) are included.

transcription factors, including an ethylene-responsive transcriptional coactivator-like protein and Drm4, plus glutathione *S*-transferase 19 (**Sup. Table 2**).

Immature ears, though fully shielded from UV-B photons, also respond differently to UV-B irradiation of canopy leaves if plants were UV-B acclimated in field conditions, or grown in the greenhouse in the absence of UV-B.[11,14] As shown in **Figure 2C**, 458 transcripts showed differential expression when comparing immature ear (IE) transcriptomes from 2L IR greenhouse plants to IE from 2L IR field plants. 87 transcripts showed at least 2-fold increased levels and 371 decreased levels in plants under greenhouse versus field conditions ($p < 0.05$, **Fig. 2C and Sup. Table 3**); 22% of the differentially increased transcripts in the greenhouse samples correspond to transcription factors, suggesting that with just a 4 h exposure time, subsequent changes in ear development and physiology will be extensive. More differences exist in the downregulated genes; these are transcripts that show higher expression in immature ears from plants that were acclimated at the field. It is interesting, but not unexpected, to note that many transcripts for proteins participating in stress responses, such as enzymes in oxidative stress detoxification (Class III peroxidases and glutathione S-transferases) and heat shock proteins (HSPs 17.2, 17.9, 70, 82, 90; **Sup. Table 2**) show higher expression levels in immature ears from field plants than from greenhouse plants. We conclude that field plants are better acclimated to UV-B and to other environmental variables and more readily express higher levels of transcripts associated with acclimation to UV-B. Collectively, these comparisons demonstrate that numerous transcripts are regulated differently in plants acclimated by natural UV-B versus greenhouse plants exposed to UV-B irradiation in a single treatment after maturing in the absence of UV-B.

Proteome changes. Two-dimensional DIGE and mass spectrometry were used to identify differentially accumulated proteins.[11,12,16] **Figure 3A** shows that, of the 58 total protein spots that were changed by the 4 h UV-B treatment in 2L IR greenhouse leaves compared to the NI control plants, only 24% were changed in a similar way in 2L IR field leaves. Common proteome changes include the upregulation of some photosynthetic proteins (fructose-bisphosphate aldolase, pyruvate phosphate dikinase, chlorophyll a-b binding protein 1) and a cytosolic ascorbate peroxidase; as well as the downregulation of other photosynthetic proteins including both RUBISCO and an oxygen-evolving enhancer protein 1 (**Sup. Table 4**). For immature ears, 70 protein spots were changed by UV-B in greenhouse plants,

of which 34% were also changed in ears from acclimated field plants; in this shared set, two protein spots were increased and 22 decreased (**Fig. 3B**). The shared increased proteins are a cytosolic glyceraldehyde-3-phosphate dehydrogenase and an unknown protein, while the group of similarly decreased proteins includes stress response proteins, such as a [Mn] superoxide dismutase, several ascorbate peroxidases, a DNA repair protein RAD23 and several unknown proteins (**Sup. Table 5**). As described for transcriptomic changes, the majority of proteomic changes elicited by UV-B exposure are distinctive in plants acclimated to natural UV-B compared to greenhouse plants exposed to a single UV-B treatment.

Metabolome changes. Finally, as provided in detail in **Figure 4** under the two growth conditions, IR and SH leaves showed distinctive metabolites. Previously, we found that under greenhouse conditions, UV-B radiation induced changes in the levels of 30 metabolites.[11] In this new set of experiments, twenty eight of these 30 metabolites also showed significant differences in field samples (**Fig. 4**). Alanine, fructose, galactose, glucose, xylose, dihydroascorbic acid dimer, 2-ketoglutamic, shikimic and quinic acid, were all modulated in the 2L IR vs. SH greenhouse samples,[11] and are also significantly regulated in field samples (**Fig. 4**); however, absolute concentrations are different in the two growth conditions after the same 4 h UV-B treatment (**Fig. 4**). Some metabolites that previously showed different levels in IR versus SH leaves[11] such as glucaric/galactaric acid, dopamine and trans-caffeoylquinic acid were almost undetectable in field samples (**Fig. 4**). These metabolites likely reflect "acute" responses to mild UV-B in the greenhouse, and are unlikely to be signal molecules synthesized in irradiated field leaves and transported to shielded organs to coordinate systemic responses. Other sets of metabolites were UV-B-regulated and were changed in both irradiated and shielded leaves; interestingly, all metabolites changed in both organs in the greenhouse were modulated in the field leaves, but most had a different absolute level (**Fig. 4**). These molecules are more likely candidates for signals in systemic responses.

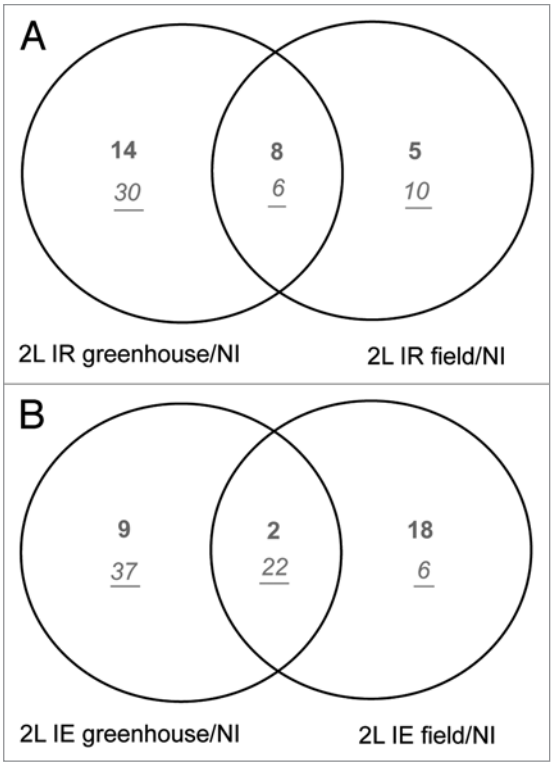
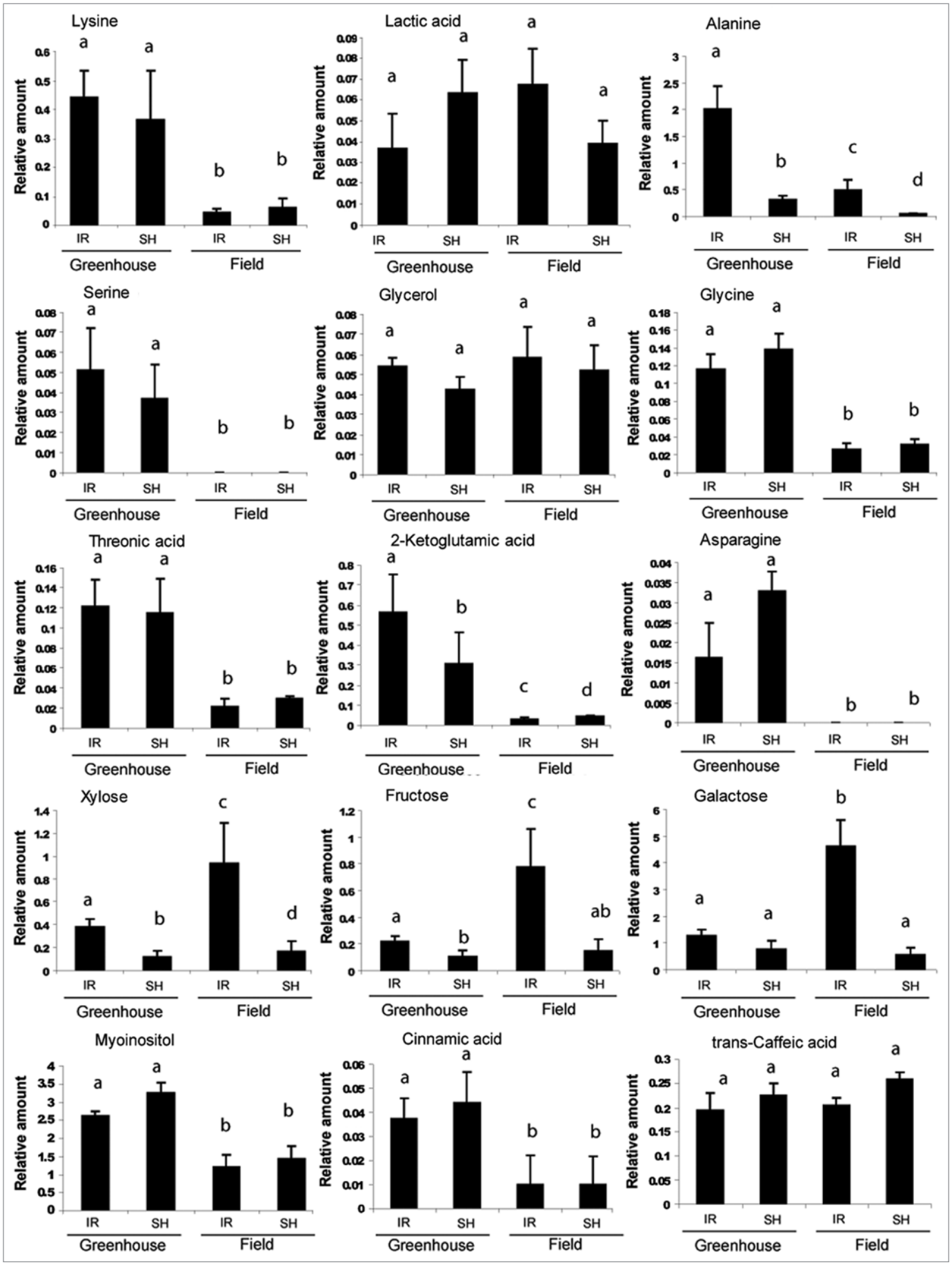


Figure 3. Proteome changes in the two irradiated canopy leaves receiving supplementary UV-B (2L IR) and immature ears in the greenhouse compared to field plants. Increased proteins are red in bold; decreased proteins are green, underlined in italics. (A) Intersection of proteins changed in irradiated leaves from greenhouse plants with two irradiated leaves (2L IR) and field plants with two canopy leaves receiving supplementary UV-B, compared to non-irradiated plants (NI). (B) Intersection of proteins changed in immature ears from greenhouse plants with two irradiated leaves (2L IE) and field plants with two canopy leaves receiving supplementary UV-B, compared to non-irradiated plants (NI). Proteins changed by 1.5-fold ($p < 0.05$) are included.

Discussion

UV-B regulates diverse processes involved in acclimation to the damage it causes, including increasing expression of genes involved in UV protection such as UV-absorbing phenolic compounds that act as epidermal UV-B sunscreens,[17,18] light-stimulated repair genes such as DNA photolyases,[19] and other genes for damage repair.[20] Importantly for plants, UV-B is not only an agent of damage, but is a key environmental signal that can modulate physiology and development in organs not directly exposed to solar radiation. With terrestrial levels of UV-B increasing, reflecting continued instability in stratospheric ozone, it is important to further our understanding of plant responses to UV-B exposure and to include real world settings.

Most UV photobiology studies in plants have been carried out in controlled environments using irradiation protocols in greenhouse or growth chamber conditions where no UV-B is present prior to the defined treatment period. These conditions cannot occur in natural environments where plants are constantly exposed to UV-B from solar radiation and consequently become acclimated to UV-B. To better understand the extent to which single UV-B treatments under greenhouse conditions elicit responses that parallel maize responses under field conditions, we conducted microarray experiments, analytical protein 2D gel analysis and mass spectroscopy to identify differentially accumulated proteins and metabolite profiling by GC-MS. Transcript, protein and metabolite abundances in irradiated adult leaves, shielded adult leaves and immature ears were assessed under both greenhouse and field conditions. The absolute number of transcript differences is higher in the naïve greenhouse plants than in acclimated field plants, and most of the spectrum of differentially expressed transcripts was distinctive. Common elements include transcripts for genes involved in sunscreen biosynthesis and some regulators. We conclude that greenhouse and field plants show substantially different responses to a single 4 h UV-B treatment. Similarly, we found that the majority of proteins abundances affected by UV-B were distinctive to either field or greenhouse conditions. Prior acclimation to UV-B results in fewer transcript and protein losses and metabolite changes. The single irradiation treatment exaggerates the impact of UV-B compared to the


Lysine
Lactic acid
Alanine
Serine
Glycerol
Glycine
Threonic acid
2-Ketoglutamic acid
Asparagine
Xylose
Fructose
Galactose
Myoinositol
Cinnamic acid
trans-Caffeic acid
Relative amount
IR
SH
Greenhouse
Field

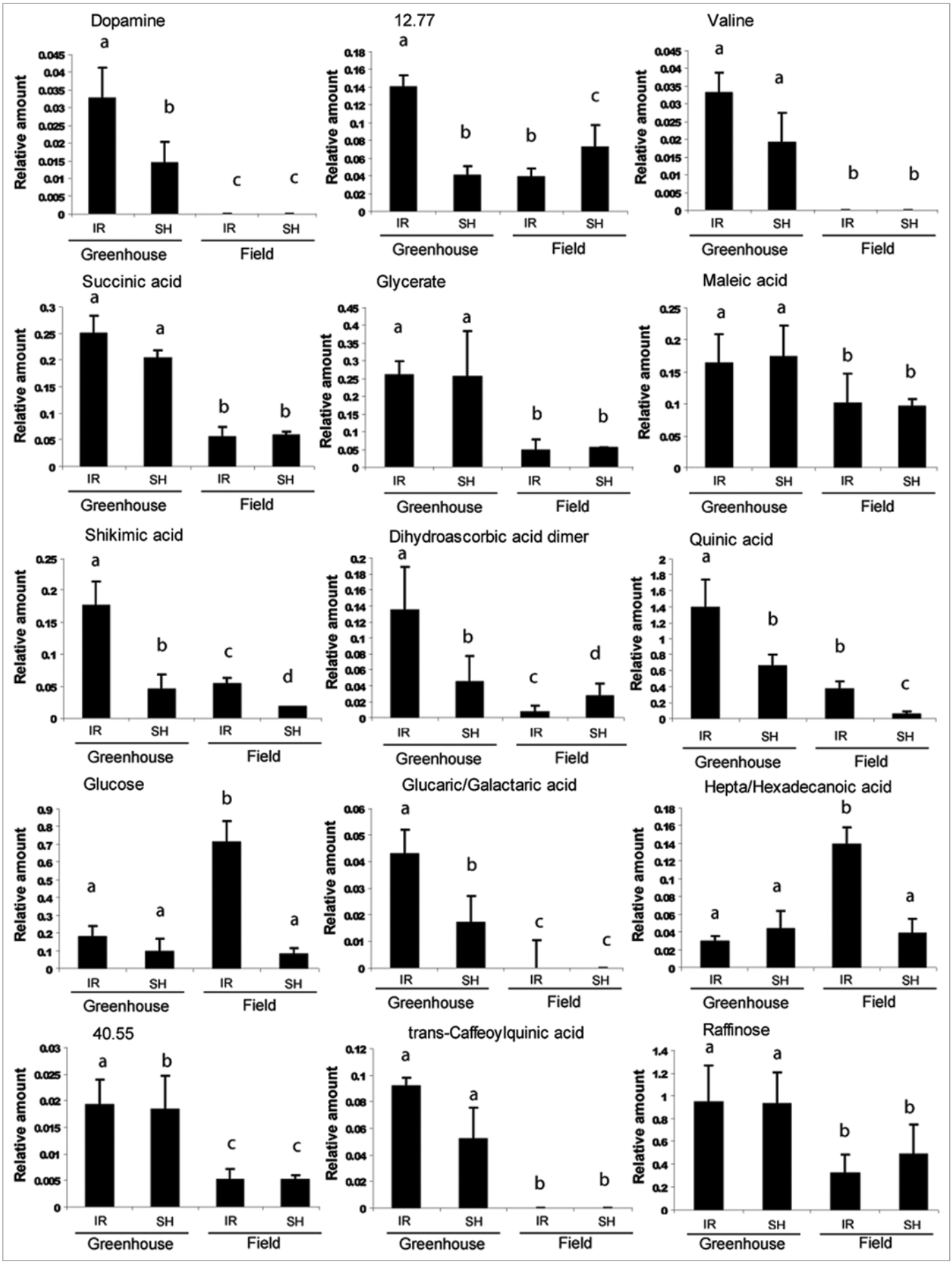

Dopamine
12.77
Valine
Succinic acid
Glycerate
Maleic acid
Shikimic acid
Dihydroascorbic acid dimer
Quinic acid
Glucose
Glucaric/Galactaric acid
Hepta/Hexadecanoic acid
40.55
trans-Caffeoylquinic acid
Raffinose
Relative amount
IR
SH
Greenhouse
Field

Figure 4 (see previous pages). Metabolic profiling of irradiated (IR) and shielded (SH) leaves from field or greenhouse maize plants. Only two adult leaves per plant were irradiated with UV-B during 4 h. Statistical analysis was done using one way ANOVA; statistically significant differences are labeled with letters a, b, c and d ($\alpha = 0.05$).

natural world. Although most metabolites were present in both greenhouse and field-grown leaves, absolute levels of metabolites were distinctive. As one goal of analysis of abiotic stress treatments is identifying targets for amelioration through plant breeding or transgenic technology, any discovery based solely on analysis of plants grown without UV-B to one or a few discrete UV-B exposure regimes needs to be validated in plants grown under solar conditions to determine whether affected genes and processes are of consequence under natural growing conditions.

Materials and Methods

Treatments. Inbred W23 *bz2* (defect in sequestration of anthocyanin in the vacuole) was grown for 5 wks in the greenhouse or at the field during summer 2008 using the same protocol as described previously in reference 21. The afternoon prior to treatment, 16 greenhouse pots were transferred into a wire grid beneath UV lamps (TL 20 W/12; Phillips, Eindhoven, The Netherlands) at a UV-B intensity of 2 W/m^{-2} and a UV-A intensity of 0.65 W/m^{-2}. The bulbs were covered with cellulose acetate filters (100 mm extra clear cellulose acetate plastic, Tap Plastics, Mountain View, CA); the cellulose acetate sheeting does not remove any UV-B radiation from the spectrum but excludes wavelengths lower than 280 nm. The lamps were mounted to a wooden platform and raised ~1.7 m above the floor and ~0.5 m above the canopy; the topmost two leaves were threaded through a slit in PE plastic suspended below the lamps; this plastic absorbs UV-B. After recovery from manipulation overnight the plants received a 4 h UV-B exposure. In the field, the same UV apparatus was moved over a set of plants planted in a similarly spaced grid, and the top two leaves threaded through the PE plastic the afternoon before the supplementary UV-B treatment. Greenhouse plants that were fully irradiated and a set of plants receiving no UV-B were used as controls; as with the treatment groups, these pots were moved and aligned under the lamps the afternoon prior to treatment. After treatment, four plants were pooled as a replicate, and the three sample types (irradiated leaf, shielded leaf and immature ear) collected, placed in 50 mL plastic tubes and immediately flash-frozen in liquid nitrogen. Dissected immature ears were cut in half and randomized as to which half (upper or lower) went into separate tubes within replicates.

Microarray experiments. RNA extraction and microarray hybridization on a custom Agilent 4 x 44K platform were done as described in Casati and Walbot (2008).[22] Data acquisition, image processing and spot flagging and removal were done as described in Skibbe et al. (2009).[12] Data analysis was performed as described in Casati et al. (2011).[11] Microarray data was deposited in GEO under ID Series GSE25038.

Protein extraction and 2D gel electrophoresis. Protein extraction, labeling, 2D gel electrophoresis, gel image analysis, MS and database searches were done as described in Falcone-Ferreyra et al. (2010).

Metabolite profiling. Extraction, liquid partition and derivation prior to GC-MS analysis were performed as described by Casati et al. (2011).[11]

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Note

Supplemental materials can be found at:
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