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# Distinctive transcriptome responses to adverse environmental conditions in *Zea mays* L.

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## Summary

Maize seedling transcriptome responses to six abiotic perturbations (heat, cold, darkness, desiccation, salt, ultraviolet-B) were analysed. Approximately 7800 transcripts were expressed in one or more treatments compared with light-grown seedlings plus juvenile leaves from field-grown plants. Approximately 5200 transcripts were expressed in one or more treatments and absent in light-grown seedlings. Approximately 2000 transcripts were unique to one treatment. Salt and heat elicited the largest number of transcript changes; however, salt resulted in mostly a decreased abundance of transcripts, whereas heat shock resulted in mostly an increased abundance of transcripts. A total of 384 transcripts were common to all stress treatments and not expressed in light-grown seedlings; 146 transcripts were present in light-grown seedlings and absent from all stress treatments. A complex pattern of overlapping transcripts between treatments was found, and a significant pattern of congruence in the direction of transcript change between pairs of treatments was uncovered. From the analysis, it appears that the scope of gene expression changes is determined by the challenge, indicating specificity in perception and response. Nonetheless, transcripts regulated by multiple responses are generally affected in the same manner, indicating common or converging regulatory networks.
The data are available for additional analysis through a searchable database.

## Introduction

Plants exist in a dynamic environment and must integrate signals of light quality and intensity, air and soil temperature, water availability and nutrient status both to maintain functional organs through physiological responses and to generate new organs appropriate for the prevailing conditions. Abiotic and discrete chemical treatments are characterized by exemplar changes in gene expression, such as the induction of heat shock proteins (Iba, 2002). With the advent of transcriptome profiling, it is now possible to document the full scope of RNA abundance changes and then to address the two opposing models of how plants respond to environmental perturbations.

One view is that integrated plant responses to environmental fluctuations are achieved through a limited set of interconnected key pathways (Anderson *et al*., 1995). Results from yeast provide strong evidence for the centralized integration and response regulation of a subset of key genes. Transcriptome analysis after diverse abiotic challenges has demonstrated that more than one-half of the yeast genome is involved, summing across all treatments; importantly, about 10% of the genes (almost all under the control of the *Msn2/Msn4* activators of responses to abiotic perturbations) show differential expression in all stress treatments (Causton *et al*., 2001). Are there a few master stress response regulators in plants that respond to perturbations in processes such as water availability or protein unfolding to evoke identical transcriptome responses? The alternative to the central regulator hypotheses for the control of physiology and development is that both the exemplar treatment-specific genes and the majority of responses are distinct. Clearly, at some point in the translation of environmental signals into plant physiology and development, specificity in responses is required, because

the outcomes of morphological and physiological acclimations are distinctive.

The first comprehensive microarray evaluation of the central regulator hypothesis utilized *Arabidopsis* plants grown under identical conditions and then subjected to one of nine abiotic perturbations. Transcript levels were assessed for approximately 24 000 genes on Affymetrix ATH1 gene chips after 0.5, 1, 3, 6, 12 and 24 h; this large project was performed by the *Arabidopsis* Functional Genomics Network using the Col-0 ecotype. One analysis of all treatments identified 67 genes that responded to the stresses in a similar way (either up- or down-regulated), and these were differentially expressed primarily in the 1–6-h time frame (Swindell, 2006). Cell defence, energy and metabolism functional classes, rather than regulatory genes, were over-represented, lending support to the concept that changes in metabolism are key to early responses to abiotic challenges. It is possible that post-translational modification of a central regulator elicits these 67 common transcriptome changes. The major limitation of the study design was that only biological duplicate samples were employed, limiting the statistical power of the analysis. A more robust test of differential gene regulation and the identification of common and distinctive genes in the various stress treatments requires more biological replicates for each treatment.

The idea that responses can be largely independent is supported by a recent analysis of *Arabidopsis* seedling responses to seven hormones over a 3-h treatment: each hormone triggered largely independent changes of the transcriptome (Nemhauser *et al*., 2006). Although similar types of gene were triggered by some hormone treatments, different members of gene families were expressed. Transcriptome profiling studies have also been completed for individual abiotic challenges, using a variety of species, platforms and treatments. In the few studies examining several treatments on the same platform with the same tissue types, the conclusions parallel the results for plant hormones: abiotic treatments trigger unique, mostly non-overlapping responses. For example, Kreps *et al*. (2002) examined the effects of cold, salt and drought on adult *Arabidopsis* leaves and roots after 3 or 27 h of treatment. These conditions are all known to limit water availability, particularly in leaves (Salisbury and Ross, 1992). Summing the three treatments, more than 30% of the transcriptome showed a significant change relative to untreated plants, using an 8100 gene platform. At the 3-h time point, however, only approximately 5% of the transcriptome changes were common amongst the three treatments, and this decreased to approximately 0.5% on the second day. This study indicates that, in adult root and leaf, the three treatments elicit mainly different changes in the set of expressed genes, but the time resolution cannot pinpoint whether master regulators exist at the top of the hierarchy of expression, followed by treatment-specific transducers that ultimately result in discrete responses.

A particular practical reason for the analysis of stress treatments in maize arose from a consideration of strategies aimed at the generation and sequencing of full-length cDNAs (FLcDNAs) for this species. The construction of normalized libraries is expensive. To reduce the final cost whilst increasing the representation of maize transcript types, the first library constructed represented a pool of 16 developmentally distinct stages, including all of the major organs dissected from field-grown material (http://www.maizecdna.org/). To supplement coverage with a second library, we considered what additional tissue sources could add to the diversity of transcript types represented. A survey of expressed sequence tag (EST) collections from a variety of plant species indicated that environmental perturbations permitted the recovery of new suites of expressed transcript types that were either missing from or present at a reduced level in similar stage plants grown under standard conditions. Consequently, control seedlings, field-grown young plants and six abiotic challenges were selected as likely to provide a wide range of gene expression, provided that these abiotic stresses elicited discrete programmes of expression in maize. These eight samples were combined with anthers spanning the pre-meiotic, meiotic and post-meiotic stages to fill in missing developmental stages in a second normalized FLcDNA library. Deep EST sampling, using paired end reads from this normalized FLcDNA library (http://www.maizecdna.org/outreach/approach.html), confirmed that new gene types were recovered relative to the first library.

Given that the library is constructed from a complex mixture of tissues, assignment of domains of gene expression is not possible without an analysis by transcriptome profiling. Existing data permitted the annotation of the transcript types expressed in developing anthers (Ma *et al*., 2006, 2007); however, publicly available data on transcriptional responses to abiotic challenges remain very limited for maize. In the current study, four biological replicate samples from the eight physiological treatments were analysed by transcriptome profiling on a 70-mer spotted oligonucleotide array with 58 000 elements to define the expression patterns of FLcDNAs; for a validation experiment and to test the sensitivity of the 70-mer spotted array platform, a separate set of ultraviolet-B (UV-B)-treated samples was hybridized to a 60-mer *in situ* synthesized oligonucleotide platform. In addition, the data sets can be used to address the two competing hypotheses concerning the regulation of responses to abiotic challenge:

central regulators with many common responses or distinct responses for each treatment.

## Results

### Treatments

The rationale for the selection of seedling responses to environmental perturbation is that germination and plantlet establishment are crucial and irreversible phases of the maize life cycle. The seed has only limited nutrient reserves to fuel initial growth, after which the plant must become self-sufficient where it has germinated. The seedling growth rate and physiological responses to abiotic challenges may therefore be more likely than adult tissue responses to exhibit an overlapping set of responses controlled by master regulatory genes. In maize, seed imbibition, the irreversible step of germination, occurs over about 2 days and is light independent. Growth of the newly emerged shoot requires light to stimulate greening and ensure appropriate cell elongation. To determine whether seedlings respond to abiotic treatments that may be encountered in the natural environment, six conditions were tested: very low light, excess UV-B, cold, heat, salt and osmotic exposure. These treatments have well-established effects in flowering plants. Light-starved seedlings are classified as etiolated: maize leaves are cream–white and the leaf bases are extra long from excessive cell elongation, resulting in a seedling unable to stand upright (Salisbury and Ross, 1992). Although UV-B is a natural component of the environment, periodic depletion of the ozone layer causes spikes in UV-B, up to 10-fold higher than ambient levels. UV-B decreases photosynthesis and elicits the synthesis of photoprotective and repair processes (Casati and Walbot, 2003); seedlings contain higher levels of sunscreen pigments, suggesting a greater investment in UV-B avoidance (Casati and Walbot, 2005). Salt and excess external osmotic potential decrease root water uptake, slow growth and can damage cells (Hasegawa *et al*., 2000; Zhu, 2002). Cold also slows metabolism and growth (Christie *et al*., 1994), and dehydrated plants are sensitized to cold damage (Yamaguchi-Shinozaki and Shinozaki, 2006). Although maize is a heat-tolerant species, as in other plants, heat shock stimulates the synthesis of a suite of protective proteins found in all plants studied to date (Iba, 2006).

### Experiment design

To assess the diversity and possible overlap of transcriptional responses in maize during abiotic challenges, transcriptome profiling was performed on B73 inbred seedlings. The most comprehensive platform available was a 58 000-element spotted oligonucleotide array constructed by the Maize Microarray Project (http://www.maizearray.org/). The individual 70-mer oligonucleotides were designed to the Institute for Genomic Research (TIGR) maize gene index (version 13). The array contained probes for more than 40 000 sense transcript types, representing approximately 80% of the expected gene number of maize, printed on two glass slides; a subset of genes was represented by multiple probes, as has become clear from later assemblies including more ESTs and FLcDNAs. Tissues were grown at Stanford University during the summer of 2005. RNA was extracted and purified, cDNA was generated, and amplified cRNA was labelled with cyanine-3 (Cy3) or Cy5 dyes (see 'Experimental procedures' for details) from four biological replicates of each treatment. Each replicate was composed of a pool of 10 similarly treated whole seedlings (with the endosperm and kernel removed) or juvenile leaves; this pooling strategy within each biological replicate should dampen out minor differences between individuals. A fifth sample from each treatment was used in FLcDNA library construction.

As shown in Figure 1, the 32 dye-labelled cDNA samples (eight treatments × four biological replicates of each) were hybridized in a dye-balanced octagon design by the staff of the Maize Microarray Project. Data from all four replicates of one treatment sample type were considered together in evaluating the transcriptome of that sample. Two types of

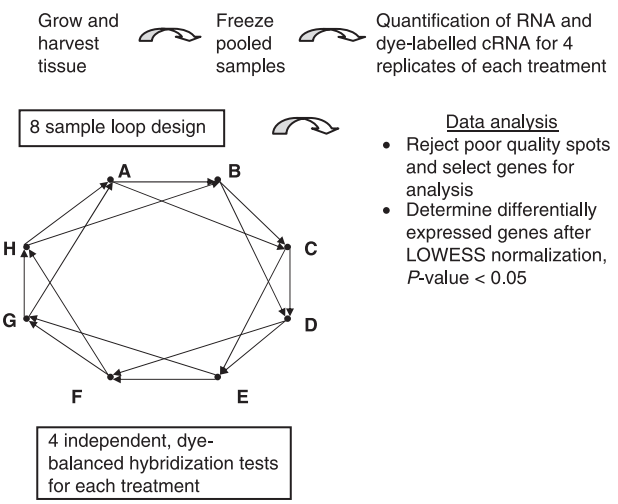



**Figure 1** Schematic diagram of the experimental design for microarray hybridization and analysis. Four replicates, each containing pooled tissues, were prepared for microarray analysis in an eight-sample loop design. In the octagon, arrowheads represent cyanine-5 (Cy5)-labelled samples and dots indicate Cy3-labelled samples in each pair of samples hybridized to the same array.

biological control were employed. The first were seedlings germinated under a constant temperature of 26 °C, with 16 h of light and adequate water (light control). The second were samples prepared from field-grown expanding juvenile leaves. Field seedlings emerged on day 6 or 7, approximately 3 days later than room-grown plants at constant temperature; juvenile field plants experienced fluctuating diurnal temperature (minimum, 11.1 °C; maximum, 31.6 °C) and light–dark cycles (maximum, 16 h of sunlight), as well as variation over 3 weeks in water availability, photosynthetically active radiation, UV-B and average daily temperature. The endosperm was depleted by approximately day 15, and the immature juvenile leaves were growing on an established plant. The field-grown sample was used to filter out transcriptional responses to modest environmental variations to highlight transcriptional responses to major perturbations in each treatment.

To generate a manageable number of samples to test the hypothesis that abiotic treatments generate unique patterns of altered gene expression, a single duration and severity was chosen for each abiotic treatment. This design is a compromise of several factors. First, treatments were designed to provide significant challenge, but with 100% survivorship of maize seedlings after return to normal growth conditions. Second, although all samples were harvested at a chronological age of 84 h, treatments altered growth parameters and hence seedlings were of different sizes in the various treatments. This problem of matching samples by chronological time (rather than by developmental age) is intrinsic to treatments that perturb growth, even when many time points are evaluated. Third, the treatments differed in length, primarily because some treatments induce rapid physiological changes throughout the exposed tissue (heat shock, UV-B on leaves), whereas others require a longer exposure time to have an impact on the plant (alterations in the root after UV-B, alterations in the leaf after salt and osmotic root shock). Heat shock was the shortest treatment (4 h at 45 °C) and induced moderate leaf wilting in the seedlings. UV-B treatment for 8 h at a fluence rate about three times higher than current ambient levels (Casati and Walbot, 2003) caused no visible damage. Cold treatment was for 24 h at 10 °C and stunted growth in both the root and shoot. Both the 250 mM NaCl and 11% w/v mannitol treatments were for 48 h and resulted in moderate leaf wilting and smaller seedlings. Near-complete darkness was imposed to achieve etiolation for the entire 84-h experiment, and this treatment resulted in cream to pale yellow seedlings that were excessively elongated in the leaf sheath and blade regions, and flopped over on to the vermiculite surface.

### Abiotic treatments elicit the expression of many new genes

The microarray data were analysed by two methods: first by the simple presence or absence of transcripts and second by the differential expression of each condition vs. the light-grown control seedling. In the first analysis, probes were marked as present for a treatment or condition if they hybridized at least 2.6 standard deviations above the average background intensity in at least three of four biological replicates. Using this criterion, 25 153 probes hybridized to at least one sample type. Of the features with detectable hybridization, 16 002 were constitutively present in all seedlings. Nearly all ($n$ = 14 955) of these transcripts were also present in the field-grown leaf samples (Table 1a); presumably most transcripts that did not hybridize with the juvenile leaf were seedling root transcripts. Because the maize genome has not yet been fully sequenced, the expression diversity values must be considered as estimates. Furthermore, redundant probes were designed from ESTs not yet assembled into the same gene models, resulting in overestimation. Countering this, ESTs have not yet been recovered for some genes, and probes for these genes were not included on the array platform. Furthermore, poorly formed (uninterpretable) spots reduced the number of features that could be evaluated and, in many cases, such signals could not be reliably distinguished from true non-hybridization signals.

Table 1a provides details of the scope of gene expression in all conditions, including the light-grown seedling and field-grown juvenile leaf. Salt treatment showed the largest number of unique transcripts ($n$ = 767), followed by heat ($n$ = 585). The number of probes shared between each sample type and any other, any two others, etc., is provided in subsequent columns in Table 1a. For example, salt shares 744 transcript types with one additional condition, 691 transcript types with two other conditions, etc.

Table 1b re-evaluates the diversity of transcriptome responses excluding all transcripts that were present in both the light seedling and the juvenile leaf controls ($n$ = 16 985). A total of 7845 transcript types was present in at least one of the stress treatments and absent in both control conditions. Of the estimated 50 000 non-transposon genes in maize, these transcripts represent over 15% of the projected transcriptome, and document that abiotic challenges elicit the expression of a substantial portion of the genome. Altogether, 2090 treatment-specific transcripts were identified, about 8% of the total transcript diversity found in all of the tissues analysed (2090/25 153). Nearly 6% of the transcript types (1431/25 153) were found in all six abiotic treatments and were

**Table 1** Count of probes expressed uniquely in a treatment and shared with other treatments
(a) All treatments and probes included

| | Total treatments in which probe hybridized | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Light | 126 | 218 | 355 | 432 | 631 | 890 | 2172 | 14 955 |
| Salt | 767 | 744 | 691 | 818 | 1011 | 1148 | 2269 | 14 955 |
| Dark | 84 | 275 | 416 | 614 | 798 | 977 | 2197 | 14 955 |
| Desiccation | 49 | 143 | 245 | 479 | 730 | 881 | 2076 | 14 955 |
| UV-B | 139 | 244 | 340 | 539 | 816 | 993 | 2233 | 14 955 |
| Cold | 122 | 236 | 409 | 576 | 792 | 1009 | 2218 | 14 955 |
| Juvenile leaf | 197 | 166 | 252 | 301 | 448 | 555 | 1313 | 14 955 |
| Heat | 585 | 412 | 478 | 493 | 724 | 957 | 2042 | 14 955 |

Probes found in a specific treatment only in the first column (column 1), in that treatment and one other (column 2), in that treatment and two others (column 3), etc.

(b) Comparison of abiotic stress treatments after removal of probes expressed in both light-grown seedling and field-grown juvenile leaf

| | Abiotic stress treatments in which probe is present | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| Salt | 854 | 904 | 768 | 872 | 973 | 1431 | |
| Dark | 93 | 388 | 524 | 728 | 916 | 1431 | |
| Desiccation | 51 | 172 | 327 | 689 | 922 | 1431 | |
| UV-B | 191 | 297 | 427 | 639 | 856 | 1431 | |
| Cold | 147 | 357 | 507 | 594 | 793 | 1431 | |
| Heat | 754 | 482 | 480 | 478 | 605 | 1431 | |
| Probes | 2090 | 1300 | 1011 | 1000 | 1013 | 1431 | 7845 |

Probes found in a specific treatment only in the first column (column 1), in that treatment and one other (column 2), in that treatment and two others (column 3), etc. The last column provides the number of transcript types common to all six treatments.

(c) Comparison of abiotic stress treatments with probes expressed in light-grown seedling removed

| | Total abiotic stress treatments in which probe hybridized | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| Salt | 785 | 704 | 511 | 536 | 503 | 384 | |
| Dark | 85 | 278 | 311 | 426 | 454 | 384 | |
| Desiccation | 51 | 151 | 241 | 441 | 495 | 384 | |
| UV-B | 183 | 270 | 342 | 463 | 498 | 384 | |
| Cold | 136 | 298 | 394 | 422 | 436 | 384 | |
| Heat | 652 | 351 | 325 | 264 | 259 | 384 | |
| Probes | 1892 | 1026 | 708 | 638 | 529 | 384 | 5177 |

Same as Table 1b, except that the probes expressed in the light-grown control seedling were removed.

absent from both controls. Once again, salt and heat exhibited the largest numbers of unique transcript types, by nearly a factor of four compared with the next treatment (UV-B, 191). Collectively, these data indicate that the majority of transcriptome responses are unique to a treatment; however, there is a large core (18% of total responses, 1431/7845) that is common to all treatments, despite the difference in lengths of treatment, which ranged from 84 h of darkness to 4 h of heat.

Table 1c shows an analysis of the transcripts excluding the probes marked as present in the light-grown control seedling

**Table 1** (*Continued*)
(d) Comparison by treatment: singular, paired and multiple treatments

| Treatment | Salt | Dark | Desiccation | UV-B | Cold | Heat |
|---|---|---|---|---|---|---|
| Salt | 854 | 3371 | 3094 | 3000 | 3001 | 2733 |
| Dark | 280 | 93 | 2913 | 2784 | 2655 | 2328 |
| Desiccation | 86 | 20 | 51 | 2692 | 2587 | 2278 |
| UV-B | 116 | 41 | 25 | 191 | 2562 | 2312 |
| Cold | 183 | 18 | 16 | 33 | 147 | 2318 |
| Heat | 239 | 29 | 25 | 82 | 107 | 754 |

The values above the diagonal represent the number of probes expressed in the two indicated treatments (row and column headings) plus at least one additional treatment. The diagonal indicates the number of probes expressed in only a single treatment. Below the diagonal, the number of probes expressed in the two indicated treatments is given. The data for the juvenile leaf and light-grown seedling were removed prior to the analysis to focus on the genes expressed only in the acute stress treatments.

($n$ = 19 779). The number of transcripts present in at least one stress treatment decreased to 5177, whereas 384 transcripts remained present in all treatments.

Table 1d provides more detail concerning the overlap between treatments, using the same data set as for Table 1b (excludes probes present in both light seedling and juvenile leaf). In Table 1d, the diagonal represents treatment-specific responses (same values as column 1 of Table 1b). Values below the diagonal are shared by only the two treatments indicated by the row and column headings, and the values above the diagonal sum the results for the two indicated treatments plus one or more additional treatments. For example, only 239 of the differentially expressed transcripts are shared between heat and salt. The salt treatment shares 183 transcript responses with cold, another desiccating treatment; this is 25% more than the transcripts unique to the cold response, but less than one-fourth of the complexity of the salt-specific response programme. The categories shared by three or more treatments are all large, from 2278 to 3371 transcripts, demonstrating that many stress-modulated transcripts show differences after three or more distinctive treatments. This trend may explain why treatments commonly considered as similar – such as cold and desiccation – share only 16 transcript types expressed only in these treatments, whereas 2567 transcripts are shared by cold, desiccation and at least one more treatment. A detailed listing of transcript counts shared with every combination of abiotic treatment is provided in Table S1 (see 'Supplementary material'). All combinations of shared transcripts were detected, albeit some at very low numbers: i.e. only five transcripts were expressed in dark, desiccation and heat treatments, but were undetectable in salt, UV-B and cold treatments (Table S1).

Another test of the relatedness of treatments is the construction of an unrooted tree. From a diagram showing the similarity between treatments, it is clear that each is distinctive, despite sharing many transcripts with other treatments (Figure 2). This tree utilizes the data solely for the transcripts present after abiotic treatments, i.e. not expressed in either the light-grown control seedling or field-grown juvenile leaf. A tree generated from differential expression data relative to the light-grown control seedling was nearly identical (not shown).

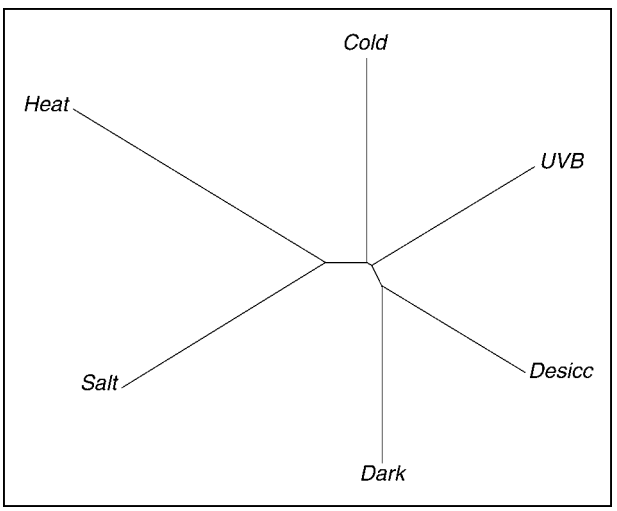



**Figure 2** Unrooted tree linking the treatments based on transcripts absent in both control seedlings and field-grown juvenile leaf, but present in a treatment.

Considering the global patterns of maize seedling responses to abiotic treatments, the results indicate that complex transcriptome changes are elicited, each treatment has a unique signature and there is every possible pattern of overlap between treatments. The extensive sets of overlapping suites of genes found in every pattern of shared expression are the major contributors to the dispersed nature of the unrooted tree (Figure 2).

### Classification of transcripts by gene ontology (GO) terms

One aim of this work was to identify pathways affected by specific abiotic stresses and to find genes that were regulated only in certain stress conditions. Table S2a,b (see 'Supplementary material') summarizes the plant processes affected after the different treatments, based on the identification of GO terms. The EST sequences originally used for probe design were matched to a 'grasses-only' Uniprot protein database, and the resulting proteins were sent to the AgBase server (http://www.agbase.msstate.edu) for GO annotation. EST sequences without a protein match were then sent to the AgBase server for GO annotation via sequence similarity. Of the 40 000 sense transcripts, approximately 26 000 showed a BLAST hit in the Uniprot grass database: 11 270 of these probes were expressed in at least one treatment (representing 8645 unique Uniprot IDs), and 7106 showed a molecular function type GO term based on a cut-off value of $1 \times 10^{-15}$ (representing 5538 unique Uniprot IDs). This data set, representing about one-third of the total probes expressed in at least one sample, was used to analyse which GO terms were over-represented under each individual stress condition or in a pair of treatments only.

Because various GO terms represent different levels of detail (e.g. 'ion binding' including the more specific 'magnesium ion binding' at a deeper level), terms that were two levels more specific than the top-level 'molecular function' term were labelled as 'Level 3' terms for a starting point. Table S2a shows that 541 terms were assigned to a subset of the 854 probes expressed uniquely after salt stress. The functional Level 3 terms with the largest number of genes correspond to 'transferase activity' (90), 'nucleotide binding' (84), 'hydrolase activity' (80) and 'nucleic acid binding' (72). Because the Level 3 terms are general, a more specific classification was performed in Table S2b. Thus, in the major 'transferase activity' group, distinctive transferase activities are included, as represented by enzymes of general metabolism, such as 6-phosphofructokinase, acyltransferases and alanine transaminase; this category also includes regulatory enzymes, such as protein kinases, protein methyltransferases, etc. In particular, there are 20 protein serine/threonine kinases that are expressed only after salt stress; this suggests that unique signal transduction pathways are induced that are not activated when plants are under other stresses, such as desiccation.

Heat stress is the group with the next largest number of GO term classifications (327 probes). As shown in Table S2a, for this stress, 'nucleotide binding' is the group with the largest number of genes (66), followed by 'hydrolase activity' (54), 'transferase activity' (49) and 'nucleic acid binding' (39). In the 'nucleotide binding' group, 50 probes correspond to ATP binding proteins, whereas four are GTP binding proteins (Table S2b). It is important to note that, after salt and heat stress, there were 72 and 39 uniquely expressed 'nucleic acid binding' proteins, respectively. This group includes a number of transcription factors that are probably activated under these individual stress conditions, and are therefore candidates for tracing the activation of specific pathways of stress responses. Similarly, for the other stresses, 18 uniquely expressed nucleic acid binding proteins were identified in the UV-B samples, 19 under cold stress, seven in dark conditions and three after desiccation (Table S2a). In addition, there are specific nucleic acid binding proteins that are expressed in two treatments; these may be involved in generating the observed pattern of overlap between treatments. To compare across treatments, the percentage contribution of each Level 3 term towards the total number of terms assigned to the treatment-specific probes is shown in Table 2. The highest percentages within a treatment are given in bold, showing that the 'hydrolases' term is highly represented in every stress. This group includes various types of enzyme, such as ATPases, proteases, sugar degradation enzymes, phosphatases and nucleases (Table S2b). Although this group includes very different enzymatic activities, it is again important to note that, even if the same types of activity are present for each treatment, different genes encoding similar activities are induced by individual stresses. Until more genes can be assigned GO terms, the several terms unique to a treatment cannot be considered as a complete and accurate representation, but may be a starting point for further analyses.

To further aid in the classification of responses, the translated EST sequences originally used for probe design were BLASTed against *Arabidopsis* protein sequences, and the resulting hits corresponding to probes present in all stresses, but absent in both the light seedling and the juvenile leaf controls, were analysed using the AraCyc OMICS Viewer tool at the *Arabidopsis* website (http://www.arabidopsis.org:1555/expression.html). This tool places expression data on to well-established biochemical pathways to permit the visualization of whether entire pathways or specific branches of metabolism are co-ordinately regulated. Overall, there were a number of enzymes in many pathways that were expressed only under abiotic stresses (Figure S1, see 'Supplementary material'). Figure 3 zooms in on the cytosolic glycolysis pathway effects. As shown in Figure 3, most of the enzymes are in gene families with at least three members. Individual family members can be expressed only under stress conditions, responding to all stresses to a similar extent (Figure 3a, in colour), but not expressed (or expressed below detection

**Table 2** Gene ontology (GO) annotation analysis of genes expressed in a single treatment, showing the percentage of each 'Level 3' term over all 'Level 3' terms found

| Level 3 GO term | Salt | Dark | Desiccation | UV-B | Cold | Heat |
|---|---|---|---|---|---|---|
| Amine binding | 0.2% | | | | | |
| Carbohydrate binding | 0.9% | 2.8% | | | | 1.2% |
| Chromatin binding | 0.4% | | | | 2.1% | |
| Cofactor binding | 1.8% | | 6.7% | 3.2% | 2.1% | 1.5% |
| Drug transporter activity | 0.4% | | | 1.1% | | |
| Enzyme activator activity | 0.6% | | 6.7% | | | 0.6% |
| Enzyme inhibitor activity | | | | | | 0.9% |
| Helicase activity | 0.9% | | | 1.1% | 7.4% | 1.8% |
| Hydrolase activity | **14.8%** | **16.7%** | **13.3%** | **21.5%** | **13.7%** | **16.5%** |
| Ion binding | 11.3% | **22.2%** | **20.0%** | **18.3%** | 8.4% | 7.3% |
| Isomerase activity | 1.1% | | 6.7% | | 3.2% | 1.2% |
| Ligase activity | 1.5% | 2.8% | | | | 2.4% |
| Lipid binding | 0.2% | | | | | |
| Lyase activity | 0.2% | | | 1.1% | 1.1% | 1.2% |
| Metal cluster binding | 0.2% | | | | | 0.3% |
| Microtubule motor activity | 0.6% | | | | | |
| Nucleic acid binding | 13.3% | **19.4%** | **20.0%** | 8.6% | **20.0%** | 11.9% |
| Nucleotide binding | **15.5%** | 5.6% | | 10.8% | **18.9%** | **20.2%** |
| Oxidoreductase activity | 4.4% | 5.6% | 6.7% | 7.5% | 3.2% | 2.4% |
| Protein binding | 8.1% | 2.8% | | 4.3% | 8.4% | 6.4% |
| RNA polymerase II transcription factor activity | | 2.8% | | | | |
| Signal transducer activity | 3.3% | 2.8% | | 2.2% | 2.1% | 5.5% |
| Structural constituent of cell wall | | | 6.7% | | | |
| Structural constituent of ribosome | 0.9% | 2.8% | | 2.2% | | 1.2% |
| Substrate-specific transporter activity | 1.7% | 2.8% | | 1.1% | | 1.2% |
| Tetrapyrrole binding | 0.9% | 2.8% | | 5.4% | | 0.9% |
| Transferase activity | **16.6%** | 8.3% | **13.3%** | **11.8%** | 9.5% | **15.0%** |
| Transmembrane transporter activity | 0.2% | | | | | |
| Total terms | 541 | 36 | 15 | 93 | 95 | 327 |

The top three percentages for each treatment are indicated in bold.

levels) in control conditions. Examples of isozymes expressed only under specific stress conditions are shown in Figure 3b (heat) and Figure 3c (salt): one isoform of glyceraldehyde-3-phosphate dehydrogenase (GapC-1) is only expressed under heat stress, and two phosphofructokinases (homologues to the *Arabidopsis* proteins AT1G12000 and AT2G22480) and a phosphoglycerate mutase (similar to AT1G09780) are specific to salt stress. As expected, there are other members for each gene family (in black) that are expressed constitutively or under different growth conditions. This pathway analysis demonstrates both the expression of stress-specific members of gene families and the complex overlap between stress responses.

## Abiotic challenges result in both increased and decreased transcript abundances

Thus far, the analysis has focused on the presence or absence of a transcript; however, as shown in Figure 4, the treatments are also distinctive in the extent to which the environmental perturbation causes the up-regulation or down-regulation of transcripts that are expressed in the control samples. As shown in Figure 4a, charting genes with twofold or greater changes in abundance ($P < 0.05$), many genes were differentially regulated by a stress treatment, compared with the light-grown control seedling. What is striking here is that salt treatment, with the largest number of transcript changes, is primarily a response of down-regulation of transcript abundance (84% of altered transcripts were decreased). In contrast, the response to heat involves 86% up-regulation of transcript abundances. Other treatments, such as cold and UV-B, showed nearly equivalent numbers of up- and down-regulated transcripts. With the more relaxed criteria of a 1.5-fold change (Figure 4b), about twice as many transcript types were found to be differentially regulated.

To further define the set of down-regulated transcripts, Table 3a shows the transcripts absent in all stress treatments,

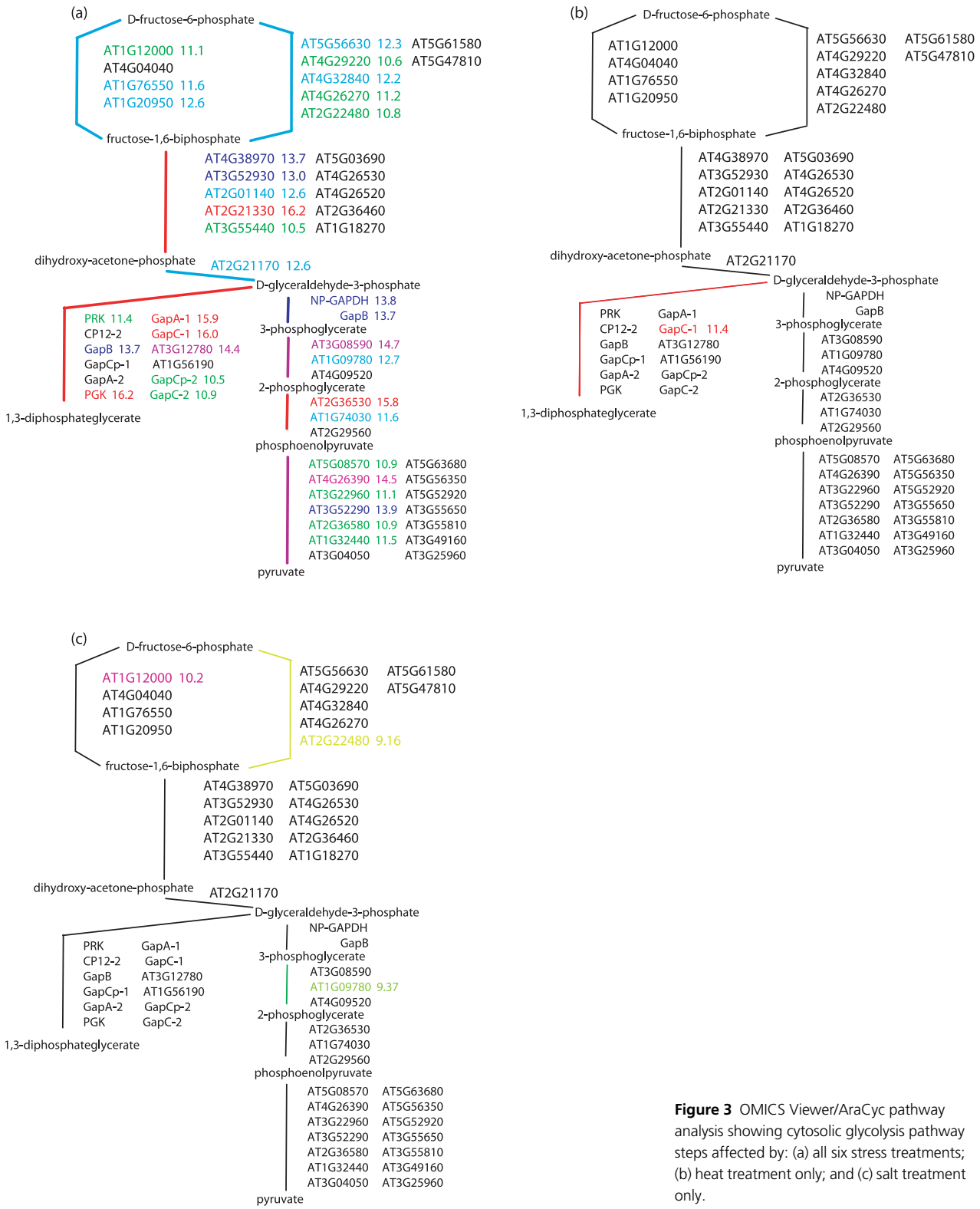



**Figure 3** OMICS Viewer/AraCyc pathway analysis showing cytosolic glycolysis pathway steps affected by: (a) all six stress treatments; (b) heat treatment only; and (c) salt treatment only.

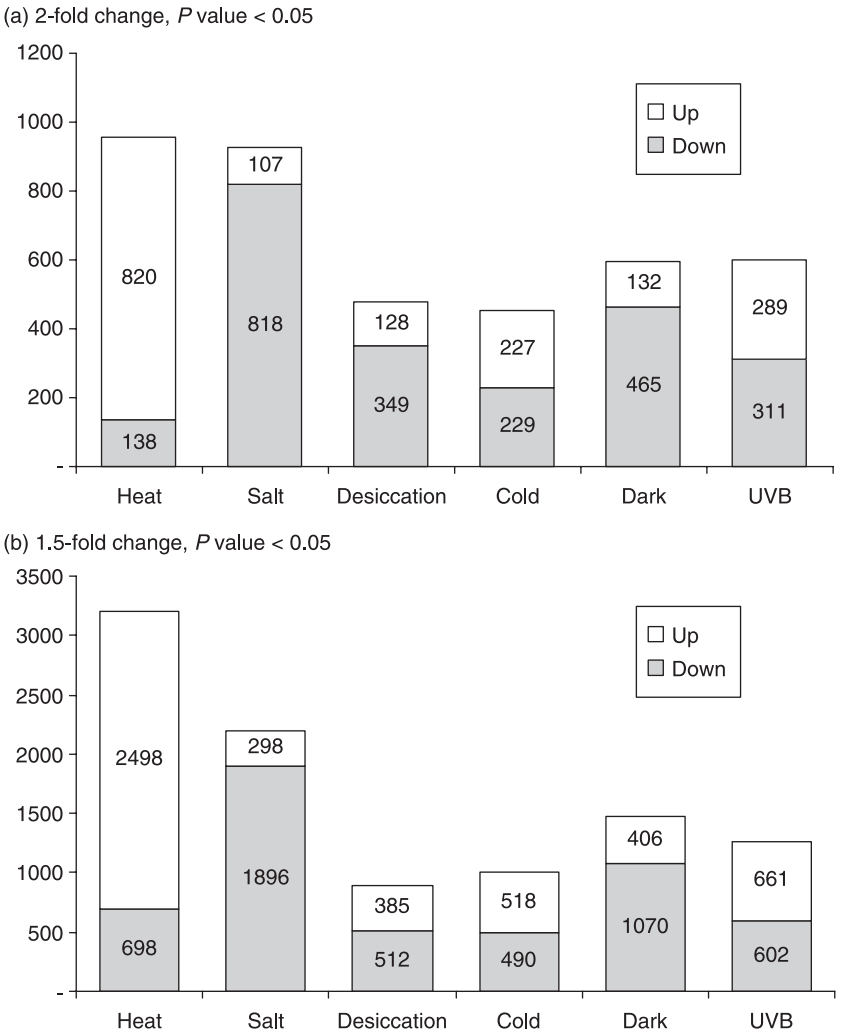



**Figure 4** Stacked bar charts showing number of transcripts up- or down-regulated in comparison with the light-grown seedling: (a) twofold change cut-off; (b) 1.5-fold change cut-off.

but present in the light-grown seedling ($n = 126$), the field-grown juvenile leaf ($n = 197$) or both of these control samples ($n = 20$). For the common class, there is enrichment for genes associated with photosynthesis (Table 3b).

These analyses led to the question of whether specific genes are regulated in the opposite manner by the treatments. To address this question, each pair-wise combination of treatments was analysed for shared gene expression, classified as down, up or no change. A meta-analysis of these data is presented in Table 4. The first entry shows congruent changes (up–up or down–down, for example). It is striking that 78% of genes expressed in any two treatments are either differentially regulated in the same direction or not differentially expressed in these treatments. The second entry shows non-congruent combinations. The majority (14%) of these represent cases of differential regulation in one treatment and no change in the other treatment. Only 8% of cases reflect opposite patterns of expression: down in one treatment and up in another. All pair-wise combinations of treatments are presented in Table S3 (see 'Supplementary material). The striking result is that both up, both down and no change in both categories comprise the majority of shared transcripts for all types of stress. Very few transcripts are up-regulated in one treatment and down-regulated in a different treatment, i.e. down in salt, up in dark ($n = 1$) or up in salt, down in dark ($n = 4$). The largest divergent class was down in salt, up in heat ($n = 9$). Therefore, the abiotic challenges tested trigger changes in transcript abundance in the same direction (up or down) when differentially expressed transcripts are shared between treatments. This insight suggests that shared regulatory pathways activate or repress transcript abundances, probably acting through transcriptional rate changes for genes involved in common stress responses. If true, then the specificity of which genes are expressed is a separate (and

**Table 3** Probes not present in stress treatments

(a) Probes called present in the light-grown seedling or juvenile leaf controls, but not present in any stress treatment

| Light | Juvenile leaf | Probes (*n*) |
|---|---|---|
| No | Yes | 197 |
| Yes | No | 126 |
| Yes | Yes | 20 |

(b) List of 20 probes not present in any stress treatment, but present in both controls

| Probe ID | Putative annotation |
|---|---|
| MZ00001562 | N/A |
| MZ00004394 | Unknown protein {*Oryza sativa*} |
| MZ00005041 | Putative Mtr/TnaB/TyrO permease family protein {*Oryza sativa*} |
| MZ00012524 | Putative protein kinase APK1B, serine/threonine protein kinase {*Oryza sativa*} |
| MZ00014363 | Bowman–Birk-type wound-induced proteinase inhibitor WIP1 precursor {*Zea mays*} |
| MZ00019327 | N/A |
| MZ00023872 | Similar to *Medicago truncatula* MtN3 (Y08726) |
| MZ00027505 | Similar to *Arabidopsis thaliana* chromosome 1, F6F9.23 unknown protein |
| MZ00034835 | Plastoquinol-plastocyanin reductase, cytochrome b6, splice form 1 – maize chloroplast {*Zea mays*} |
| MZ00034851 | Photosystem II reaction centre J protein {*Pinus thunbergii*} |
| MZ00034861 | Ribosomal protein L20 – maize chloroplast {*Zea mays*} |
| MZ00034873 | Chloroplast 30S ribosomal protein S14 {*Zea mays*} |
| MZ00038637 | N/A |
| MZ00042460 | Putative LeOPT1 – oligopeptide transporter {*Oryza sativa*} |
| MZ00043400 | Hypothetical protein – garden asparagus {*Asparagus officinalis*} |
| MZ00044027 | N/A |
| MZ00049369 | OSJNBb0059K02.24 {*Oryza sativa*} |
| MZ00054986 | At3g57190 putative protein {*Arabidopsis thaliana*} |
| MZ00055890 | N/A |
| MZ00056319 | Photosystem I P700 chlorophyll A apoprotein A2 (PsaB) (PSI-B) {*Zea mays*} |

**Table 4** Meta-analysis of the direction of transcript abundance changes – all pairs of treatments compared

| Expression change | Probes |
|---|---|
| Same direction | 4411 |
| Opposite direction | 454 |
| Up/down ↔ 'no change' | 813 |

See Table S3 for a complete listing of all pair-wise comparisons between treatments.

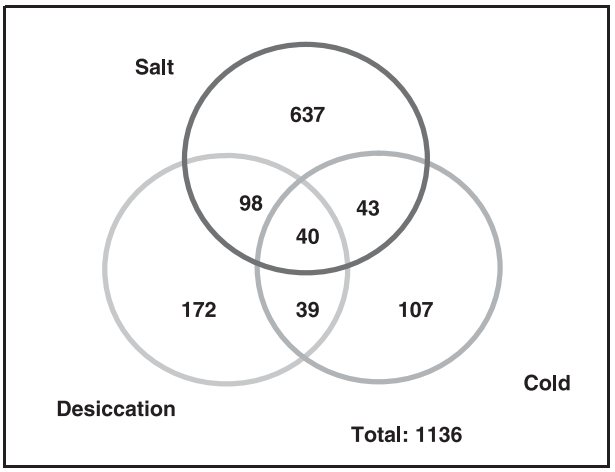
preceding) step that determines the scope of the response. Within that scope, the outcome on a gene-by-gene basis is almost always the same (up or down) from all responses.

Because of the common water deprivation effect, differentially regulated transcripts for the salt, desiccation and cold treatments were compared using Venn diagrams (Figure 5a, down-regulated; Figure 5b, up-regulated). Only 40 transcripts are down-regulated (3%, 40/1136) and 17 up-regulated (4%, 17/372) in all three treatments; 40% or more of the transcripts are specific to each treatment, confirming the distinctive responses to these three treatments despite numerous pair-wise overlaps. The transcripts in common to all three treatments were spread across numerous GO categories (data not shown).

Again using the AraCyc OMICS Viewer tool, the up- and down-regulated transcripts were evaluated to confirm that particular pathways were affected by the different stresses. For example, when comparing the salt-stressed with the light-grown control plants, many enzymes involved in amino acid biosynthesis were down-regulated (Figure S2, see 'Supplementary material'), including the biosynthesis pathways for alanine, valine, methionine, aspartate, glutamine and glycine. This suggests that, under salt stress, these energy-demanding pathways are shut down to divert energy to cell recovery.

(a) Down-regulated (2-fold change) in salt, desiccation, or cold treatments




(b) Up-regulated (2-fold change) in salt, desiccation, or cold treatments

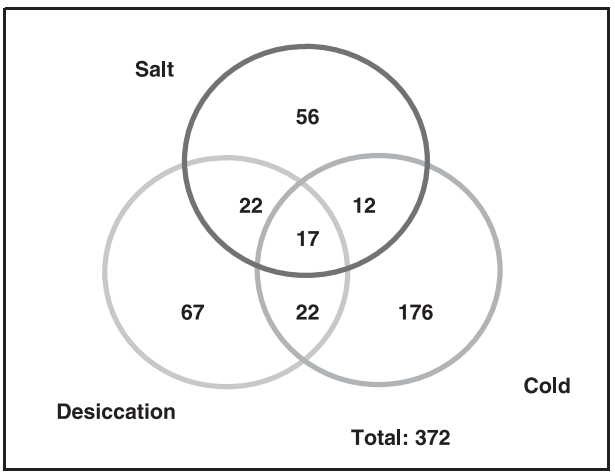



**Figure 5** Venn diagrams comparing down-regulated (a) and up-regulated (b) transcripts between salt, desiccation and cold treatments.

## Global validation experiment for UV-B

To investigate the reproducibility of the platform and to assess the robustness of the list of UV-B-activated and UV-B-repressed genes, an additional experiment was performed. Previously, the correlation between anther developmental stages analysed on a 22 000-element Agilent array and on the 58 000-element spotted 70-mer array employed in this experiment ranged from 0.5 for probes designed to distinct parts of the same gene to 0.7 for probes that were within 60 bases of each other. In that validation test, the same RNA samples were used on both platforms, eliminating any contribution of biological variability. For the current validation experiment, the UV-B-treated seedlings and field-grown juvenile leaves experiencing solar UV-B were compared with a new experimental treatment. One-week-old glasshouse-grown B73 plants were exposed to an 8-h UV-B treatment and expanding seedling leaves were harvested immediately afterwards. The resulting cRNA samples from four biological replications (each containing a pool of harvested leaves to minimize between plant variability) were hybridized to a 44 000-element Agilent array containing 60-mer *in situ* synthesized oligo probes, in comparison with control plants that received no UV-B.

**Table 5** Validation using Agilent arrays – probes designed within 60 bases of each other

| Arizona | Agilent | Probes |
|---|---|---|
| Absent | Absent | 1765 |
| Present | Present | 2562 |
| Absent | Present | 1984 |
| Present | Absent | 201 |
| | | 6512 |

Based on probes designed to within 60 bases of the same gene.

As shown in Figure 6, the expressed genes are plotted as log-2 values to compare the data from the Agilent platform with both the UV-B-treated seedlings (8 h treatment) (Figure 6a) and field-grown juvenile leaves receiving daily solar UV-B (Figure 6b). The correlation is 0.59 for the comparison of seedlings with UV-B-treated glasshouse leaves and 0.64 for the comparison of field-grown juvenile leaves with untreated glasshouse leaves.

As shown in Table 5, 66% (1765 + 2562) of the 6512 probes designed within 60 bases of each other were called absent on both or present on both platforms. This validation experiment highlighted a significant limitation to the comparison of Agilent arrays with the spotted 70-mer oligo platform: 1984 probes called as present on the commercial platform were not detectable on the spotted oligo array. Eighty-five per cent of the missing transcript types were in the low expression class (Figure S3, see 'Supplementary material'). Because a small subset of the low expression class has been verified by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (Casati *et al*., 2006), the spotted oligo array is considered to be less sensitive in detecting the rarer transcript classes. This limitation, combined with the instances of poorly formed spots, reduced the number of comparisons between the two platforms significantly. Nonetheless, the subset of genes confirmed as expressed in seedling and juvenile leaves after UV-B treatments of B73 maize represents a core of the universal leaf responses to this challenge, and

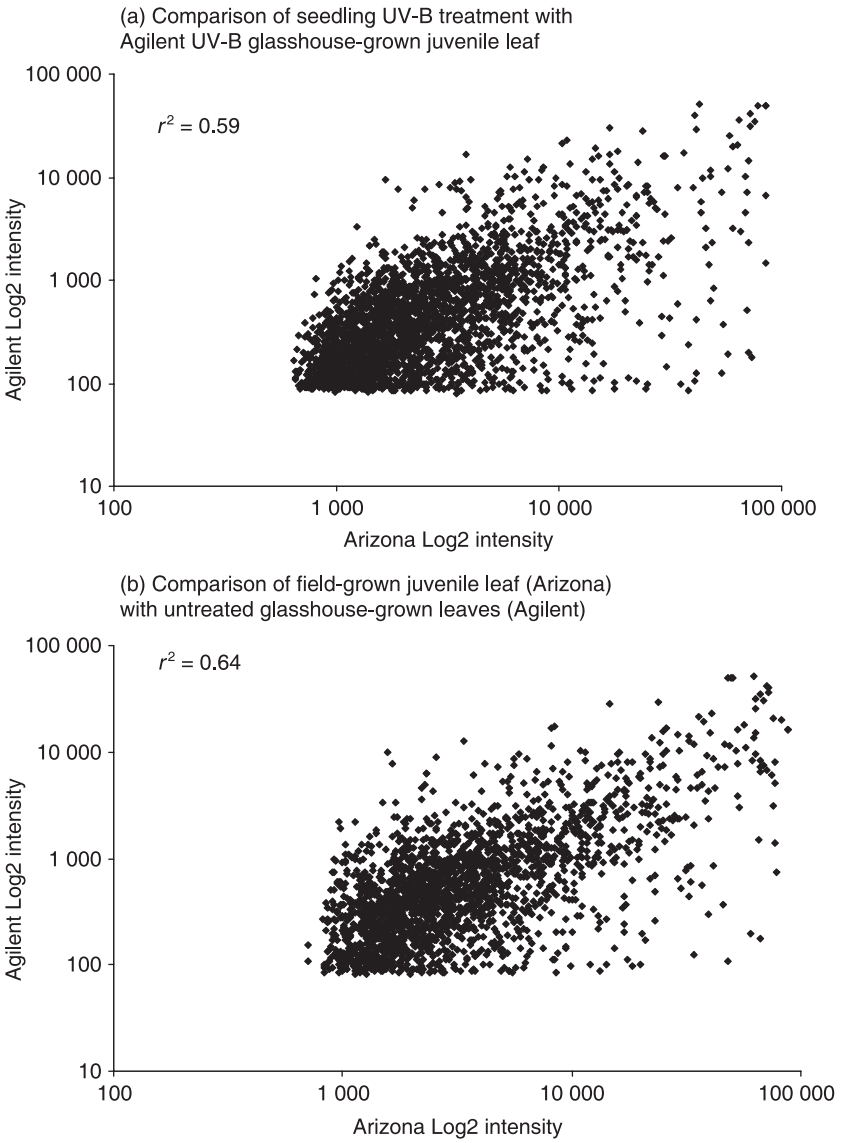



**Figure 6** Global comparison of ultraviolet-B (UV-B) treatment-expressed transcripts between: (a) seedling UV-B treatment on Arizona platform with glasshouse-grown UV-B juvenile leaf on Agilent platform; and (b) field-grown juvenile leaf on Arizona platform with untreated glasshouse-grown leaves on Agilent platform.

greatly expands the list available from the profiling of B73 on a spotted cDNA platform (Blum *et al*., 2004).

## Discussion

The initial rationale for the maize transcriptome profiling experiments performed here was to justify the construction of a FLcDNA library, i.e. to ask whether the six abiotic treatments induced a diverse set of new genes that were not normally expressed in 7-day-old seedlings. The answer to this question is unequivocally 'yes', because 5177 new transcript types were detected in the six stress treatments relative to the light-grown seedling control, and more than 7000 transcripts present in the stress treatments were missing from both the light-grown seedling control and field-grown juvenile leaves. Tissues from these two normal growth conditions were included in the first normalized library sampled by the Maize FLcDNA Project; therefore, it was important to determine whether abiotic treatments would elicit novel transcript types for the construction of a second library.

The data from this study also greatly extend the stress transcriptome results for maize, expanding the data sets based on printed cDNAs or on the recovery and analysis of a limited number of FLcDNAs from treated tissues (Jia *et al*., 2006). The maize abiotic treatment experiments have generated lists of genes differentially expressed after a specific treatment or treatments in B73 maize, the background genotype for both the full genome and FLcDNA sequencing projects. Once the genome is completed, the data can be used to interrogate promoter sequences for motifs common to genes expressed in specific patterns, as a first step in identifying functional regulatory motifs for maize stress-responsive genes. The

complete transcriptome profiling data will be used to annotate where and when individual FLcDNAs are expressed (http://www.maizecdna.org/). In addition to the Gene Expression Omnibus (GEO), the data derived from the spotted 70-mer arrays are available in a searchable database (Zeamage; http://www.maizearray.org/maize_study.shtml); queries can be constructed to evaluate specific treatments or genes.

Of the interesting trends observed, salt elicited the most distinctive suite of genes in a 48-h treatment, far more than either cold or osmotic treatments of the same duration, although these treatments are considered together as cases of water deprivation (Salisbury and Ross, 1992). Interestingly, most (84%) of the differentially regulated genes represented cases of significant down-regulation of transcript abundance after salt treatment. The second most diverse set of unique transcripts was generated by a 4-h heat shock treatment and, in this case, most transcripts were up-regulated.

Analysis of the seedling transcriptome changes indicates that plant responses are complex and involve thousands of maize genes, about 10% of the probable maize gene number (5177/50 000). The two prevailing models of plant response to abiotic challenge are as follows: (i) activities are integrated through a few master sensors and regulators; or (ii) each treatment elicits a discrete set of responses because it is coordinated through a stress-specific sensor and regulatory system. The maize data presented here indicate that the stress treatments share many elements in common: 1431 transcripts were expressed in all six treatments, representing 18% of the total response (1431/7845) when considering transcripts present in one or more stress treatments but absent in both the light-grown seedling and juvenile leaf controls. Focusing on the light-grown control seedling only, 384 transcripts were present in one or more stress treatments and absent in the control, and 146 transcripts were present in the control seedling and absent in all of the stress treatments. These 530 common responses to abiotic challenges point towards the existence of some shared regulatory mechanisms.

However, the treatment-specific transcripts constituted higher percentages: 27% (2090/7845) when considering both types of control; 37% (1892/5177) when considering the comparison of stress treatments with the light-grown control seedling. It is possible that detailed time-course experiments would reclassify many apparently treatment-specific transcripts as common genes; our treatments included short (heat, UV-B), day-long (cold) and multi-day (salt, desiccation, etiolation) treatments. Of great interest for future experiments are the GO categories that were over-represented in specific treatments. For example, after the 2-day salt treatment, there were 20 protein serine/threonine kinases expressed that were not detected in the other stress treatments. It is probable that at least some of these kinases participate in salt-specific signalling and metabolic changes, and represent a target gene type for the manipulation of maize responses to this abiotic challenge. A number of putative transcription factors were also identified that were uniquely expressed in one abiotic challenge, and may confer aspects of treatment-specific gene expression patterns. It was also found that metabolic pathway enzymes were affected in different ways depending on the stress situation, but there were some common enzymes that responded to a similar extent to all stresses. This was the case for some enzymes participating in non-photosynthetic energy generation. Glycolysis is the process responsible for the conversion of monosaccharides to pyruvic acid: this pathway is ubiquitous in cellular metabolism. Although the majority of glycolytic enzymes are common to all organisms, glycolysis in higher plants possesses numerous distinctive features (Plaxton, 1996). Plant glycolysis occurs in both the cytosol and plastids. Cytosolic glycolysis is a complex regulated network containing alternative enzymatic reactions. The cytosolic glycolytic network may provide an essential metabolic flexibility that facilitates plant development and acclimation to environmental stress. Thus, the use of different isoenzymes after a stress situation, as measured in our study, suggests that this is one regulatory mechanism for this pathway.

The core of common transcript types in maize is much larger than the apparent number in *Arabidopsis* (67/26 000) evaluated across nine abiotic challenges (Swindell, 2006). An important difference in the experimental design was the inclusion of both a standard seedling growth treatment for maize and field-grown leaves that experienced daily changes in environmental conditions. By filtering against both types of control, the maize data could focus on transcript changes that exceeded 'normal' environmental fluctuations. For *Arabidopsis*, nearly all of the common responses were identified within the first few hours of treatment, whereas, in maize, the common core of transcript types was present despite the wide differences in treatment length. It is somewhat surprising that maize responses from 4 to 84 h of treatment shared so many transcripts in common: 1431 called present relative to transcripts absent in both controls, and 530 called present or absent relative to the light-grown seedling only. The difference between the species could reflect a fundamental distinction in the perception and processing of environmental signals by maize and *Arabidopsis*, perhaps as a result of strong selection for robust growth during maize domestication (Wright *et al*., 2005; Yamasaki *et al*., 2005). The inclusion of more types of abiotic challenge for maize might reduce

the number of common transcript types; however, it seems unlikely that the number would decrease from 1431 (about 3% of the genes distinctive to both the seedling and juvenile leaf) or 530 (about 1%) to a proportion as low as that found in *Arabidopsis* (0.3%) when a comprehensive platform to test the expression of all maize genes becomes available.

In considering the hypotheses of shared sensor/regulators for all stresses or of distinctive elements for each stress, there are two key findings from these maize experiments. First, although each treatment elicits changes in a unique suite of genes, there is a complex pattern of shared transcripts between pairs or multiple treatments. In an unrooted tree, each treatment appears to be very distinct (Figure 2); however, examination of the shared patterns (Tables 1, S3; Figure 4) indicates that particular stress treatments overlap extensively and in every possible pattern. Therefore, there are unique pathways dedicated to specific stress responses, but there are also complex intersections and 'cross-talk' in common metabolic pathways between the stress responses. Second, our results help to clarify the logic of stress responses. Because treatments of different duration were compared, we did not expect to find early-acting master regulators that could be specific to each stress treatment. Nonetheless, it is clear that, when a transcript type changes in one treatment (either up or down), responses in other treatments are nearly always (78%) in the same direction (Table 4). Therefore, the scope and specificity of responses to abiotic challenges may be treatment specific, but, for the vast majority of genes whose transcripts are altered by two or more treatments, the outcome is similar. Therefore, if a gene (or transcript type) is altered during a stress response, it is probable that the same regulatory pathways (or pathways that converge quickly) will result in the up- or down-regulation of core genes in all treatments.

Beyond the initial physiological responses to abiotic stress, plants also alter the development of new organs in response to current environmental conditions. Cellular, organ and organismal developmental responses are coordinated by a limited suite of hormones and ions. Parallel to the hypothesis of master physiological regulation, one hypothesis is that hormone effects are interdependent because they regulate master growth-determining genes, such as the nuclear repressor DELLA proteins, to achieve integrated growth control (Achard *et al*., 2006). Appropriate physiological and developmental outcomes may be coordinated by calcium signalling, which is triggered by diverse treatments as a prelude to acclimation (Knight, 2000); metabolite signals, such as calcium, may elicit similar transcriptional changes in a core set of central regulators that, in turn, alter physiology quickly and development on a longer time scale. In the future, it will be interesting to use transcriptome and proteome profiling to discover whether common outcomes to stress treatments, such as leaf curling to avoid water loss or excess UV-B, are mediated by common or distinctive regulators and protein constituents.

## Experimental procedures

### Plant material and treatments

Maize inbred B73 seed (gift of Pioneer stock UT703 23 C30L) (Pioneer Hi-Bred International, Inc., Johnston, IA, USA) was propagated in the field for two generations by self-pollination, once at UC-Berkeley by Mike Freeling and then at Stanford University. For the juvenile leaf sample, 3 × 6-cm strips of blade from expanding leaves 6 or 7 from 3-week-old juvenile plants were removed in the 2005 field; samples from 10 individuals were pooled and frozen in liquid nitrogen to make one sample. For all other treatments, whole seedlings were used. To generate the seedling samples, eight self-pollinated ears were shelled, mixed and random seed was selected. Sets of 140 seeds were dusted with Green Light Systemic Fungicide (Green Light Co., San Antonio, TX, USA), and then planted, 2 cm deep, in 5 cm of sterilized vermiculite; seven rows were sown transversely in 25 × 51-cm plastic trays with bottom drainage holes to prevent waterlogging. Trays were saturated with distilled, deionized water and then placed under fluorescent illumination (16 h at 350 $\mu E/m^2$ from high-output, cool white F24T12 CW-HO bulbs; GE, Cleveland, OH, USA) on racks in a walk-in room at 26 °C. For a subset of treatments, planted trays were started in the dark to accommodate the treatments during final light treatment, as outlined below. Water equilibrated to the same room temperature was added on the morning of the third, fifth, sixth and seventh days, except as indicated. Several treatments were conducted weekly. For the control sample, seedlings were maintained for 84 h in the growth room. For the etiolation treatment, a tray was covered with three layers of heavy duty aluminium foil for the 7-day growth regime and water was added on days 3 and 6 only. For the salt treatment, one tray was watered on the final 2 days with 250 mM NaCl to saturation, resulting in a 48-h exposure. For the desiccation treatment, one tray was watered with 11% w/v mannitol to saturation on the final 2 days, resulting in a 48-h exposure. For three treatments, a tray was moved during the last day to a Percival 30B growth chamber with a 16-h/8-h light/dark schedule; illumination was provided by a mixture of fluorescent (GE F24T12 CW-HO) and incandescent (GE 40T10) bulbs at 500 $\mu E/m^2/s$. For the heat treatment, seedlings were exposed to 45 °C for 4 h during the light treatment, corresponding to the last 4 h of the 84-h regime. For the cold treatment, one tray was exposed to 10 °C for 24 h, with the final 8 h in the dark. For the UV-B treatment, seedlings received 8 h of supplementary UV-B (FB-T1-110A; Fisher Scientific, Indiana, PA, USA) during the final light illumination. Tissue harvest was performed by removing an entire seedling, clipping off the kernel, washing the vermiculite off (at 10 °C for the cold treatment and 45 °C for the heat shock treatment) and then freezing in liquid nitrogen in a labelled coin envelope. Groups of 10 seedlings from the same treatment were pooled to make a single sample. Arbitrarily, four of these pools were utilized as the biological replicates in the microarray hybridization experiments. An additional set of samples was used to construct a full-length cDNA library: one set was sent to L. Bowman (University of

South Carolina) for the characterization of small RNA populations, and one set was held in reserve.

For the validation experiment, a separate population of B73 plants was grown in the glasshouse using a combination of sodium vapour, metal halide and blue fluorescent light fixtures that simulated approximately 50% of summer solar irradiance at Stanford University. On the day before treatment, 1-week-old plants were moved to an irradiation area, and the next morning were illuminated with UV-B fixtures (Philips, F40UVB 40W and TL 20W/12) (Philips Electronics, Somerset, NJ, USA) mounted 30 cm above the canopy for 8 h. A control group of plants was also placed in the irradiation area, but was shielded from all UV-B photons by Mylar plastic (for details, see Casati and Walbot, 2003, 2004).

### Microarray hybridization and analysis

RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified by ethanol precipitation. Samples were concentrated using Qiagen MinElute columns (Valencia, CA, USA). For samples with a low concentration after this step, the volume was reduced with a Savant SpeedVac concentrator (GMI, Minneapolis, MN, USA) without heat. Samples were frozen in liquid nitrogen, and then shipped to the University of Arizona where RNA integrity, purity and quantity were measured, and the samples were labelled with either Cy3 or Cy5. Hybridization to 70-mer spotted oligo arrays followed the published protocols (http://www.maizearray.org/maize_protocols.shtml), followed by feature extraction using GenePix (Molecular Devices Corporation, Sunnyvale, CA, USA). The platform was a 58 000-element spotted 70-mer oligonucleotide array (http://www.maizearray.org/) constructed at the project based on the TIGR maize gene assembly Release 13.0; the 58 000 elements are present on two slides. During data analysis at Stanford University, many spotted oligonucleotide elements were found to be of low quality on one or more replicated array slides, limiting the ability to analyse the entire data set with all four replicates; this problem has been reported previously by Sawers *et al*. (2007), who found that only approximately 25% of the spotted oligo data were usable. Slide images were processed with GenePix software (Molecular Devices Corporation), and the resulting spot data were imported into the Limma package (Smyth, 2005) within the R software environment (http://www.r-project.org/). Unusable spots were flagged if more than 50% of the pixels were less than two standard deviations above the average background intensity, or if the GenePix 'Rgn R2' value was less than 0.5 (indicating a non-uniform spot). Spots passing these criteria were marked present. Using Limma, raw intensities were normalized within each array via the loess method and normalized between arrays using the quantile method. The average intensity for each spot with at least three usable intensity measurements was then calculated for each stress treatment. Differential expression vs. the light-grown control seedling was calculated using the 'fit' and 'eBayes' functions within the Limma package. Genes with a *P* value of less than 0.05 were indicated as differentially expressed.

All data were deposited into GEO under series ID GSE9453.

### GO classification

EST sequences were BLASTed against a Uniprot database filtered for grass species. The resulting protein hits were annotated using the AgBase (www.agbase.msstate.edu) GORetrieve tool. Unmatched sequences were submitted to AgBase's GOAnna tool for BLASTing and GO term retrieval. Output from GOAnna was filtered for values less than $1 \times 10^{-15}$ and processed to remove duplicates.

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## References

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeton, D., Peng, J. and Harberd, N.P. (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science*, **311**, 91–94.

Anderson, J.M., Chow, W.S. and Park, Y.I. (1995) The grand design of photosynthesis: acclimation of the photosynthetic apparatus to environmental cues. *Photosynth. Res*. **46**, 129–139.

Blum, J.E., Casati, P., Walbot, V. and Stapleton, A.E. (2004) Split-plot microarray design allows sensitive detection of expression differences after ultraviolet radiation in the inbred parental lines of a key maize mapping population. *Plant Cell Environ*. **27**, 1374–1386.

Casati, P. and Walbot, V. (2003) Gene expression profiling in response to ultraviolet radiation in maize genotypes with varying flavonoid content. *Plant Physiol*. **132**, 1739–1754.

Casati, P. and Walbot, V. (2004) Rapid transcriptome responses of maize (*Zea mays*) to UV-B in irradiated and shielded tissues. *Genome Biol*. **5**, R16.

Casati, P. and Walbot, V. (2005) Differential accumulation of maysin and rhamnosylisoorientin in leaves of high-altitude landraces of maize after UV-B exposure. *Plant Cell Environ*. **28**, 788–799.

Casati, P., Stapleton, A.E., Blum, J.E. and Walbot, V. (2006) Genome-wide analysis of high-altitude maize and gene knockdown stocks implicates chromatin remodeling proteins in response to UV-B. *Plant J*. **46**, 613–627.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A. (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol. Cell* **12**, 323–337.

Christie, P.J., Alfenito, M.R. and Walbot, V. (1994) Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*, **194**, 541–549.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant. Physiol. Mol. Biol*. **51**, 463–499.

Iba, K. (2002) Acclimative response to temperature stress in higher

plants: approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant. Physiol. Mol. Biol*. **53**, 225–245.
Iba, K. (2006) Trienoic fatty acids and temperature tolerance of higher plants. In: *Abiotic Stress Tolerance in Plants: Toward the Improvement of Global Environment and Food (Anonymous)*, pp. 61–68. Dordrecht: Springer.
Jia, J.P., Fu, J.J., Zheng, J., Zhou, X., Huai, J.L., Wang, J.H., Wang, M., Zhang, Y., Chen, X.P., Zhang, J.P., Zhao, J., Su, Z., Lv, Y. and Wang, G. (2006) Annotation and expression profile analysis of 2073 full-length cDNAs from stress-induced maize (*Zea mays L.*) seedlings. *Plant J*. **48**, 710–727.
Knight, H. (2000) Calcium signaling during abiotic stress in plants. *Int. Rev. Cytol*. **195**, 269–324.
Kreps, J.A., Wu, Y.J., Chang, H.S., Zhu, T., Wang, X. and Harper, J.F. (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiol*. **130**, 2129–2141.
Ma, J., Morrow, D.J., Fernandes, J. and Walbot, V. (2006) Comparative profiling of the sense and antisense transcriptome of maize lines. *Genome. Biol*. **7**, R22.
Ma, J., Duncan, D., Morrow, D.J., Fernandes, J. and Walbot, V. (2007) Transcriptome profiling of maize anthers using genetic ablation to analyze pre-meiotic and tapetal cell types. *Plant J*. **50**, 637–648.
Nemhauser, J.L., Hong, F.X. and Chory, J. (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell*, **126**, 467–475.
Plaxton, W.C. (1996) The organization and regulation of plant glycolysis. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **47**, 185–214.
Salisbury, F.B. and Ross, C.W. (1992) *Wadsworth Biology Series Plant Physiology*. Belmont, CA: Wadsworth Publishing Co, Inc.
Sawers, R.J.H., Liu, P., Anufrikova, K., Hwang, J.T.G. and Brutnell, T.P. (2007) A multi-treatment experimental system to examine photosynthetic differentiation in the maize leaf. *BMC Genomics*. **8**, 12.
Smyth, G.K. (2005) *Limma: Linear Models for Microarray Data*. New York: Springer.
Swindell, W.R. (2006) The association among gene expression responses to nine abiotic stress treatments in *Arabidopsis thaliana*. *Genetics*, **174**, 1811–1824.
Wright, S.I., Bi, I.V., Schroeder, S.G., Yamasaki, M., Doebley, J.F., McMullen, M.D. and Gaut, B.S. (2005) Evolution: the effects of artificial selection on the maize genome. *Science*, **308**, 1310–1314.
Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant. Physiol. Mol. Biol*. **57**, 781–803.
Yamasaki, M., Tenaillon, M.I., Bi, I.V., Schroeder, S.G., Sanchez-Villeda, H., Doebley, J.F., Gaut, B.S. and McMullen, M.D. (2005) A large-scale screen for artificial selection in maize identifies candidate agronomic loci for domestication and crop improvement. *Plant Cell*, **17**, 2859–2872.
Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol*. **57**, 247–273.

## Supplementary material

The following supplementary material is available for this article:

**Figure S1** Full AraCyc pathway diagram showing effects in common to all six stress treatments but not to controls. Intensities from the salt treatment are used here.

**Figure S2** Partial AraCyc metabolic pathways diagram showing differential regulation of salt stress vs. the light control using log base 2 of the ratio of intensities.

**Figure S3** Probes called present in the hybridization of UV-B-irradiated seedling leaf samples analysed on the 44 000-element Agilent array platform, but not called present in the hybridization of UV-B-irradiated seedling samples evaluated on the 70-mer spotted oligo array. Probes are ranked by the intensity of the hybridization signal.

**Table S1** Numbers of probes with specific patterns of expression, either unique to a treatment or shared with two, three, four or five other treatments. 'Yes' means probe hybridized. Complete listings of gene names are available on request

**Table S2a** Gene ontology (GO) annotation analysis of genes expressed in a single treatment: 'Level 3' terms

**Table S2b** Gene ontology (GO) annotation analysis of genes expressed in a single treatment: additional detail

**Table S3** Categorization of probes as increased, decreased or unchanged ($P < 0.05$, twofold change criterion) organized by pairs of treatments. Complete listings of gene names are available on request, and see Table S4

**Table S4** Normalized intensities by treatment for each probe

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