

# The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses

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## Summary

The tolerance responses of plants to many abiotic stresses are conjectured to be controlled by complex gene networks. In the frame of the AtGenExpress project a comprehensive *Arabidopsis thaliana* genome transcript expression study was performed using the Affymetrix ATH1 microarray in order to understand these regulatory networks in detail. In contrast to earlier studies, we subjected, side-by-side and in a high-resolution kinetic series, *Arabidopsis* plants, of identical genotype grown under identical conditions, to different environmental stresses comprising heat, cold, drought, salt, high osmolarity, UV-B light and wounding. Furthermore, the harvesting of tissue and RNA isolation were performed in parallel at the same location using identical experimental protocols. Here we describe the technical performance of the experiments. We also present a general overview of environmental abiotic stress-induced gene expression patterns and the results of a model bioinformatics analysis of gene expression in response to UV-B light, drought and cold stress. Our results suggest that the initial transcriptional stress reaction of *Arabidopsis* might comprise a set of core environmental stress response genes which, by adjustment of the energy balance, could have a crucial function in various stress responses. In addition, there are indications that systemic signals generated by the tissue exposed to stress play a major role in the coordination and execution of stress responses. In summary, the information reported provides a prime reference point and source for the subsequent exploitation of this important resource for research into plant abiotic stress.

**Keywords:** AtGenExpress, bioinformatics, gene expression, microarray, abiotic stress.

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## Introduction

Plants, as sessile organisms, have evolved an enormous capacity to realize their genetically predetermined developmental programme despite ever-changing environmental conditions. Accordingly, they are able to cope with environmental conditions, including heat, drought, salinity, osmotic pressure, wounding and UV light stress. Significant progress in understanding abiotic stress responses in higher plants has been made in recent years (Shinozaki *et al.*, 2003; Xiong *et al.*, 2002). Several common aspects of the stress response have emerged. Firstly, the initiation of most abiotic

stress responses correlates with an increase in cytoplasmic calcium, in some cases with stimulus-specific oscillation patterns (Allen *et al.*, 2000; Knight, 2000; Knight and Knight, 2001; Posas *et al.*, 2000; Xiong *et al.*, 2002) and is accompanied by a transient increase in reactive oxygen species (ROS; Apel and Hirt, 2004; Mahalingam and Fedoroff, 2003). Secondly, different stress stimuli appear to induce an overlapping/common pattern of gene expression. For example, in a survey of 8100 *Arabidopsis* genes, around 2400 genes were observed as having a common expression in response

to salt, osmotic and cold stress treatments (e.g. Kreps *et al.*, 2002; Seki *et al.*, 2002). These observations suggest that a common set of signal transduction components is triggered during many stress responses. On the other hand, abiotic stimuli like, for instance, low temperature, drought and high salinity are complex. They show many different yet related characteristics, each of which may provide the plant with quite different but unique information. This multiplicity of information inherent in most abiotic stress signals reflects one aspect of the complexity of stress response pathways (Xiong *et al.*, 2002). Consequently, it is unlikely that only one sensor perceives the respective stress condition and controls all subsequent signalling events. There may be multiple primary sensors that perceive the initial stress signal (Xiong *et al.*, 2002). Secondary signals (e.g. calcium, ROS and hormones which may induce phosphorylation cascades) can initiate subsequent cascades of signalling events, which can differ from the primary reactions in a spatio-temporal way. These secondary signals may also differ in their specificity from primary stimuli, may be shared by different stress pathways and may mediate the interaction and cross-tolerance between different signalling pathways. As outputs of these signalling processes the expression of several primary transcription factors is initiated in a well-defined spatial and temporal sequence, thereby inducing specific sets of downstream stress-responsive genes. (Xiong *et al.*, 2002).

Various abiotic stresses such as wounding, heat, cold, high salinity, high osmolarity, oxidative conditions and UV light have been studied independently or in distinct combinations for their effects on global gene expression in Arabidopsis and other plant species by several research groups (Brown *et al.*, 2005; Chen *et al.*, 2002; Desikan *et al.*, 2001; Fowler and Thomashow, 2002; Hirai *et al.*, 2004; Kreps *et al.*, 2002; Rossel *et al.*, 2002; Seki *et al.*, 2001, 2002; Ulm *et al.*, 2004). However, to date there has to our knowledge been no side-by-side comparison of global transcriptional responses to all these stress treatments to test in a quantitative and kinetics way any substantial overlap of transcriptional targets among stress pathways and to unravel stimuli-specific branches of stress-induced gene expression cascades.

Coordinated by the DFG-funded Arabidopsis Functional Genomics Network (AFGN), a large-scale project for genome-wide expression profiling of the Arabidopsis wild type var. Col-0 has recently been completed (AtGenExpress). The expression profiles were generated with the Affymetrix ATH1 gene chip which allows the detection of around 24 000 protein-encoding genes (Redman *et al.*, 2004). Arabidopsis plants were treated with different environmental abiotic stress factors comprising heat, cold, drought, salt, high osmolarity, UV-B light and wounding. This study generated large data sets under well-defined, reproducible conditions. Short descriptions of the growth

and treatment conditions of the plants, the normalization and background noise adjustment of the raw data can be found in several public sources (e.g. <http://www.weigelworld.org/resources/microarray/AtGenExpress/>, <http://affymetrix.arabidopsis.info/narrays/supersearch.pl?search-terms=afgn>, <http://www.arabidopsis.org/info/expression/ATGenExpress.jsp>). The abiotic stress data set has become an extremely valuable and indispensable resource for plant functional genomics research worldwide (for a review see Bohnert *et al.*, 2006) often in connection with open-access bioinformatics tools such as the AtGenExpress Visualization Tool (<http://jsp.weigelworld.org/expviz/expviz.jsp>), Geneinvestigator (<http://www.geneinvestigator.ethz.ch>), MapMan (<http://gabi.rzpd.de/projects/MapMan>) and the Arabidopsis eFP Browser (<http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi>).

In this paper we describe the techniques and methods used to accomplish this analysis of the global transcriptome stress response in Arabidopsis. Moreover, we focus on a model bioinformatics analysis of gene expression in response to UV-B light, drought and cold stress. These studies reveal that a very fast transcriptional reaction occurs shortly after the onset of stress treatment which appears not to be stress specific and which might be induced by the activity of a core of plant environmental stress response genes. The split into stress-specific responses becomes visible at later time points, suggesting a multitude of time-resolved and specific input pathways. In addition, when a stress signal was applied to a specific organ we also observed an immediate response in untreated tissues indicating a fast-spreading systemic signal. Taken together, the information reported here provides a prime reference point for the subsequent exploitation of this important resource for plant stress research and proves the reliability and scientific value of the data sets.

## Results

### *Growth of plants before stress treatments*

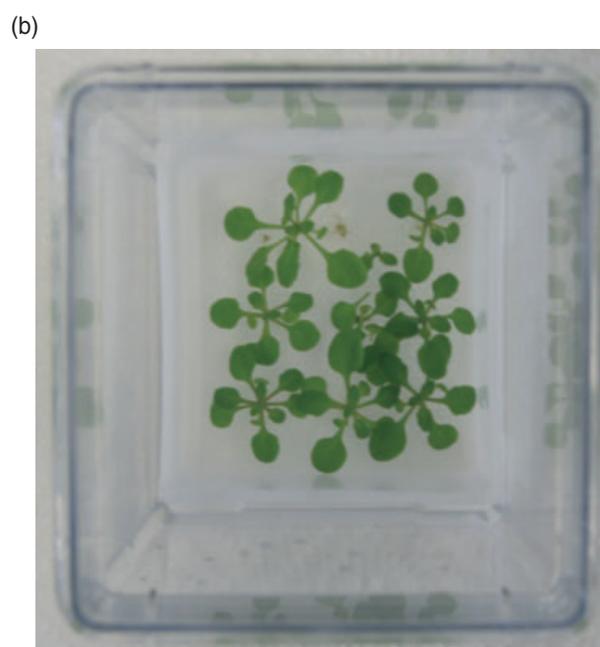
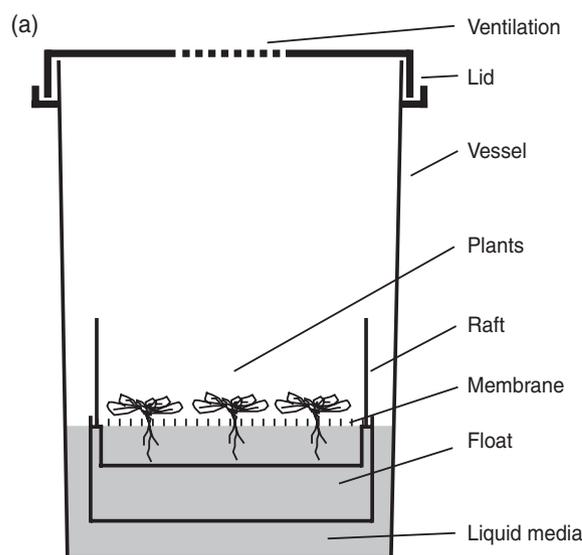
The conceptual aim of our study was to generate a sufficiently complete and time-resolved transcriptome data set to enable accurate bioinformatics determination of abiotic stress-induced gene expression in Arabidopsis. Although global transcriptional data sets are available, this approach has been limited due to differences in plant growth conditions (e.g. age of plants, light regime, growth media), stress application (e.g. transient versus continuous), harvested tissues, type of microarray used (cDNA chip, AG1, ATH1) and statistical and bioinformatics criteria (for details see Supplementary Table S1).

The methodical background of our cultivation conditions was to grow healthy plants in a way that excluded any biotic and abiotic stress and to produce a sufficient

amount of root and shoot tissue for efficient extraction of RNA. Therefore, we avoided growing plants in soil or on agar plates. Firstly, the time-consuming harvesting of clean roots out of soil or agar would have induced enormous wounding and water stress in the tissue. Secondly, the application of solutions to soil-grown plants in a spatio-temporally reproducible manner is almost impossible due to the non-homogeneous nature of soil density. We therefore decided to initially cultivate the plants for 13 days at 24°C under sterile conditions on polypropylene rafts in growth boxes containing MS medium supplemented with 0.5% agar and 0.5% sucrose (Figure 1a). Preceding tests of different cultivation procedures had revealed that the addition of sucrose to the medium was necessary during the initial incubation phase for successful cultivation of *Arabidopsis*. The boxes were closed with a lid containing an opening for air ventilation. During growth the plants were kept under long-day conditions (16 h light/8 h dark) at a light intensity of  $150 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$  and a relative humidity of 50% in a standard phytochamber. Thirteen days after sowing, the plant-containing rafts (Figure 1b) were transferred to new growth boxes containing fresh liquid MS medium without sucrose and were cultivated for five additional days under the described conditions. At this time, the plants had developed sufficient amounts of root and shoot tissue but had not initiated flowering. From several different growth regimes tested, the described growth arrangement was chosen because it ensured the most reproducible and comparable growth conditions.

#### Stress treatments

The stress treatments were initiated in parallel 18 days after sowing and 3 h after dark/light transition. The parallel performance of all stress treatments excluded differential circadian effects which could superimpose stress-induced gene expression. For stress treatments, the plants were carefully transferred from the standard phytochamber to the laboratory where all treatments were performed and then returned to the growth chamber until harvesting. For harvesting, the plants were removed from the raft and the roots were cut off. From one box only either the shoot or root material of nine plants was harvested and pooled to avoid handling stress in the other respective tissue and to minimize physiological differences between single plants. The entire harvest procedure was completed within less than 10 min. Root and shoot samples were taken in two biological replicas 0 min, 30 min, 1 h, 3 h, 6 h, 12 h and 24 h after the onset of stress treatment. In cases of expected very fast stress-induced transcriptional alterations (e.g. UV-B light) samples were also harvested 15 min after the onset of stress application. The RNA samples for control kinetics were



**Figure 1.** Principle of the cultivation of *Arabidopsis* plants for the abiotic stress treatments.

(a) Schematic representation of a cultivation vessel used for the growth of *Arabidopsis* plants.

(b) Representative picture of *Arabidopsis* plants after 18 days of cultivation. Culturing the plants on floating membrane rafts enabled simple exchange of the media and application of substances as well as fast harvest of the shoot and root tissue.

generated from non-stressed plants which were handled in exactly the same way but were not exposed to stress conditions.

Cold, heat, drought, osmotic, salt, wounding and UV-B light stress applications were performed as described below.

### *Cold stress*

The boxes containing the plants were transferred to ice for rapid cooling and kept at 4°C in the cold room until harvest. The light intensity in the cold room was 60  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ . A temperature of 3°C in the medium was reached after 30 min (Supplementary Figure S1). For this reason, the first sample was harvested 30 min after transfer of the boxes to the ice.

### *Heat stress*

The boxes were transferred to an incubator and exposed to a temperature of 38°C for 3 h and subsequently returned to the standard phytochamber until harvest.

### *Osmotic and salt stress*

The rafts containing the plants were removed from the growth boxes and mannitol or NaCl were added to the MS medium to a final concentration of 300 mM and 150 mM, respectively. The rafts were transferred back into the growth boxes and the plants were further incubated in the standard phytochamber until harvest.

### *Wounding stress*

To reduce the influence of responses related to biotic stress (Rushton *et al.*, 2002), the plants were wounded only by punctuation of the leaves with a custom made pin-tool consisting of 16 needles (about two needles  $\text{cm}^{-2}$ ). Three consecutive applications pierced an average of three to four distinct holes per leaf.

### *Drought stress*

The rafts with the plants were removed from the growth boxes and exposed to a stream of air in a clean bench for 15 min. During this time period the plants lost 10% of their fresh weight. Subsequently, the rafts were transferred back to the growth boxes, the lids closed and the plants were further cultivated in the standard phytochamber until harvest.

### *UV-B light stress*

The lids of the growth boxes were removed and the boxes covered with a 3-mm quartz plate. The plants were irradiated for 15 min with UV-B light with a biologically effective quantity of 1.18  $\text{W m}^{-2}$  (Ulm *et al.*, 2004). The UV-B light source consisted of six Philips TL40W/12 fluorescent tubes <http://www.philips.de/index.html> ( $\lambda_{\text{max}}$  of 310 nm, half-bandwidth of 40 nm; Ulm *et al.*, 2004). Afterwards the lids were closed and the boxes transferred back to the standard phytochamber until harvest. Under these conditions both

the damaging short-wavelength and the photomorphogenic long-wavelength UV-B response pathways are induced in *Arabidopsis* (Ulm and Nagy, 2005; Ulm *et al.*, 2004).

### *cRNA hybridisation and data processing*

Hybridization of cRNA using the Affymetrix AHT1 gene chip was accomplished according to a modified Affymetrix protocol developed at the Deutsche Ressourcenzentrum für Genomforschung (RZPD) in Berlin, Germany (<http://www.rzpd.de>). The raw data were imported into specialist and general data processing tools, GeneSpring and R, and were processed and normalized using the algorithms of these program packages (see also Experimental procedures). The correlation between the data of the two biological replicas of the global abiotic stress experiment was never less than 0.95, as in the examples shown for the cold, drought, UV-B light and control data sets (Supplementary Figure S2). These results demonstrate the very high reliability, reproducibility and quality of the raw data. Therefore, the mean of the results of the two biological replicas are presented in all figures and tables. The accession numbers for the array data at the Arabidopsis Information Resource (TAIR) are provided in Experimental procedures.

### *General pattern of abiotic stress-regulated gene expression*

To identify genes which are contemporaneously regulated by different stimuli we compared the gene expression patterns induced by the applied abiotic stresses.

Comparing the overall number of genes whose expression responded to one of the three treatments over a 24-h period we detected a large difference in the number of differentially regulated genes (Table 1). With the exception of high osmolarity, the number of genes whose expression was upregulated in response to the abiotic stresses exceeded the number of genes downregulated under the respective conditions (Figure 2).

The number of drought-responsive genes in our study is significantly lower than that found in the studies of Kreps *et al.* (2002) and Seki *et al.* (2002). Furthermore, the plants recovered from the drought as indicated by the transient pattern of gene expression (Figure 2). This is very likely a result of differences in stress application: Whereas Kreps *et al.* (2002) and Seki *et al.* (2002) applied a prolonged drought stress (Supplementary Table S1), the treatment in our study was transient, and thus modest. This was reflected in the expression pattern of several genes considered to be marker genes for abiotic stress (Supplementary Figure S3). Secondly, in the study of Kreps *et al.* (2002) the drought stress was applied by the incubation of the plants in 200 mM mannitol (Supplementary Table S1). This treatment also induces genes specific for osmotic stress (Figure 2; JK, unpublished results).

**Table 1** Numbers of genes differentially regulated during abiotic stress treatments

	Time points, root							Time points, shoot						
	0.25 h	0.5 h	1.0 h	3.0 h	6.0 h	12 h	24 h	0.25 h	0.5 h	1.0 h	3.0 h	6.0 h	12 h	24 h
Cold stress upregulated	ND	48	51	212	511	797	1001	ND	33	190	409	716	1030	1314
Cold stress downregulated	ND	12	11	55	81	384	826	ND	120	23	89	246	941	1328
Drought stress upregulated	110	325	309	32	131	42	6	44	141	218	217	122	129	60
Drought stress downregulated	12	42	161	16	58	23	12	59	93	21	39	22	8	14
UV-B stress upregulated	45	197	299	286	24	16	201	102	287	534	1125	1262	304	380
UV-B stress downregulated	49	40	132	305	21	7	113	55	55	84	528	743	91	70
Salt stress upregulated	ND	132	575	1263	1692	793	1310	ND	6	11	361	448	326	735
Salt stress downregulated	ND	14	240	421	1291	577	666	ND	27	22	218	187	177	494
Osmotic stress upregulated	ND	189	347	429	597	908	788	ND	34	214	797	934	1214	1553
Osmotic stress downregulated	ND	24	199	367	525	906	590	ND	23	30	325	760	1345	1832
Heat stress upregulated	41	122	468	1139	79	255	13	22	52	395	651	153	63	13
Heat stress downregulated	116	40	270	923	98	328	136	269	169	325	787	80	33	39
Wound stress upregulated	18	34	152	17	41	181	34	201	388	562	227	164	276	152
Wound stress downregulated	22	8	87	24	11	114	19	47	77	103	15	6	27	26

ND, not determined.

A comparison of the kinetics of changes in expression patterns in root and shoot tissues revealed that cold and osmotic stress induced a continuous increase in expressed genes whilst, to a different extent, salt, heat, UV-B light, drought and wounding stress caused transient alterations in gene activity (Figure 2). This observation may reflect the nature (constant versus transient) of the stress exposure. Interestingly, the number of genes responsive to drought and wounding upregulated at the 3- to 6-h time point is lower than those at 1 h and 6 h and 12 h, respectively. This indicates a biphasic response of this organ to drought stress and wounding.

Irrespectively of the type of stress applied to the plants, gene expression responded very fast to the altered environmental conditions in both roots and shoots. Significant changes in gene expression were already observed 15 and 30 min after the onset of treatment (Figure 2). Cold treatment affected the expression of a similar number of genes in both root and shoot tissue, respectively. Remarkably, the shoot-expressed genes appeared to respond more intensely over time, although the root potentially cooled down faster due to the way the plants were handled (Figure 2). Ultraviolet-B light and wounding stress were only applied to the aerial parts of the plants. However, these stresses induced fast responses in gene expression not only in the shoot but also in the root (Figure 2). The opposite was observed for salt and osmotic stress, where treatment of the roots caused significant alterations in the shoot (Figure 2).

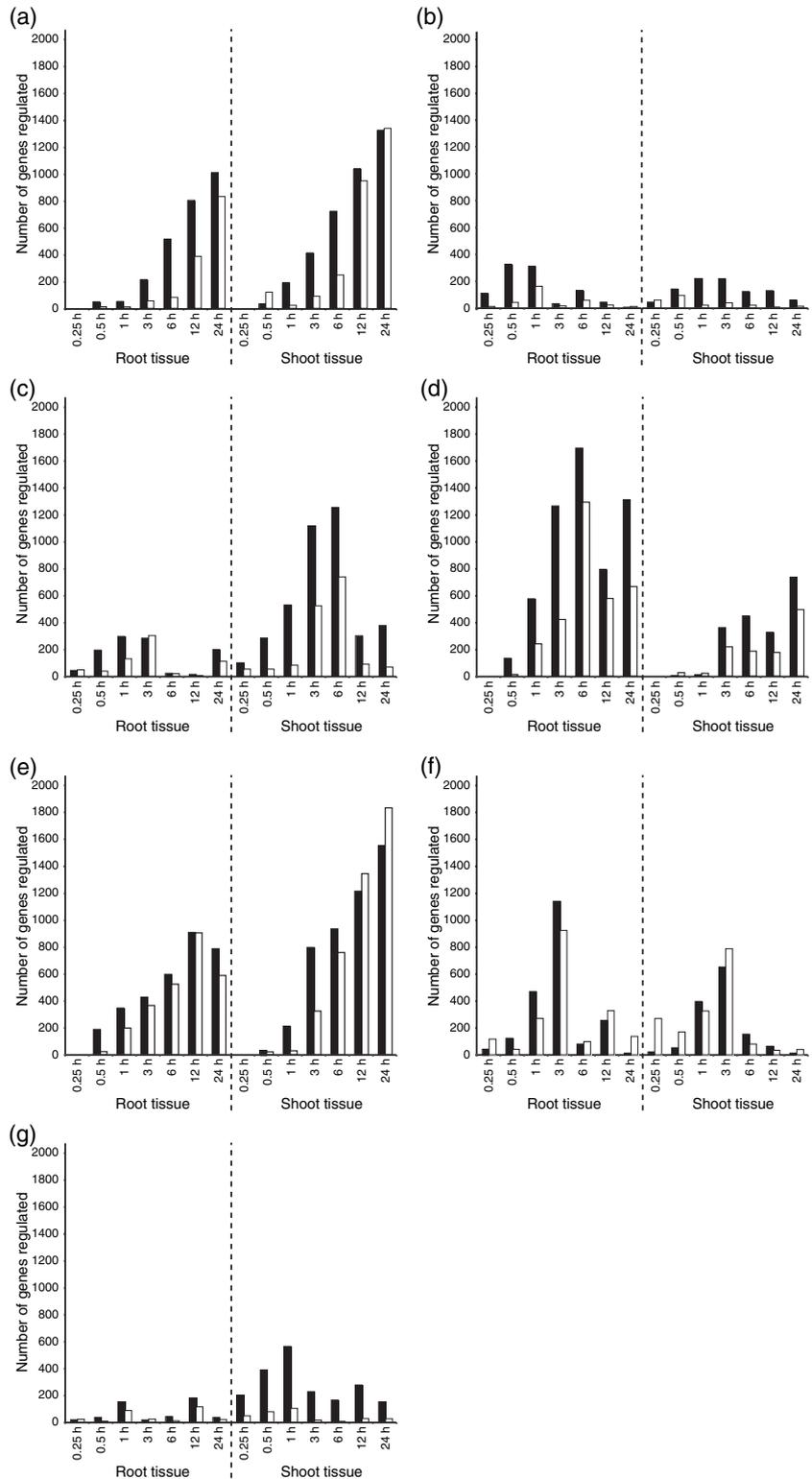
#### *Common elements of drought-, cold- and UV-B light-regulated gene expression*

We then studied drought-, cold- and UV-B light-regulated gene expression in more detail. This analysis was of particular interest because the kind of stress as well as the

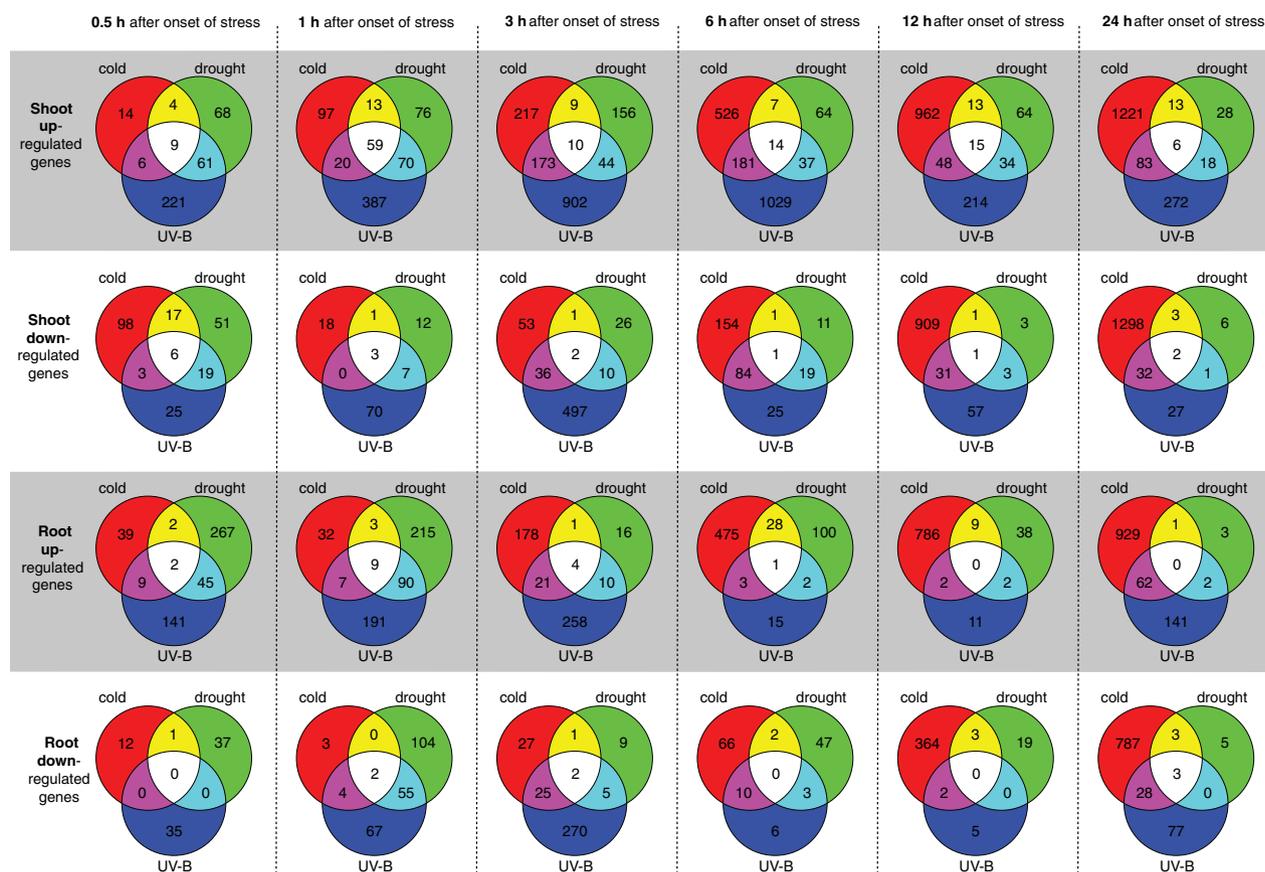
duration of the stress exposure were different (e.g. continuous, cold; transient, drought, UV-B light). Accordingly, the identification of genes commonly induced during the early response to all three stimuli may point to general and important components involved in stress-related signalling.

Response reactions to abiotic stresses like cold, drought and UV light are likely to share common second messenger molecules like  $\text{Ca}^{2+}$  or ROS. We therefore set out to investigate whether there are groups of genes which are either regulated by a specific stimulus or, alternatively, which show a transcriptional response to all stresses investigated. The latter group might represent targets of signalling components/reactions common to all responses to abiotic stresses.

As shown in Figure 3, the majority of up- and downregulated genes were specific for the applied stresses. Nevertheless, the expression of a significant number of genes was upregulated during all three different stress responses, especially at very early time points (Figure 3, Table 3, Supplementary Table S2a,b). For example, we found nine genes concertedly upregulated in the shoot 30 min after the onset of the treatment. This number increases to 59 at the 1-h time point (Figure 3). In relation to the total number of differentially expressed genes, the number of concertedly regulated genes decreased with time (Figure 3, Table 3, Supplementary Table S2). Furthermore, there appeared to be a tendency that the upregulated genes were shared to a higher degree than the downregulated genes in both roots and shoots (Figure 3). In addition, UV-B light and drought stress treatment affected the expression of a larger number of upregulated genes in a similar way in both organs at early time points (0.5–1.0 h) when compared with the other combinations of stress factors (drought versus cold, UV-B light versus cold; Figure 3). For instance, 0.5 h after stress application drought and UV-B light stress shared 70 upregulated genes in the shoot, whereas the corresponding value



**Figure 2.** Total number of genes differentially up- (black bars) and downregulated (white bars) in roots and shoots in response to cold (a), drought (b), UV-B light (c), high salt (d), high osmolarity (e), heat (f) and wounding (g) stress treatment. The tissues for RNA extraction were harvested at the indicated time points. For data normalization, processing and statistical analysis as well as for the classification of genes to be up- or downregulated see Experimental procedures.



**Figure 3.** Specificity and interference of genes regulated in response to cold, drought and UV-B light stress in shoots and roots. The Venn diagrams depict data of all time points after stress application (0.5–24 h). The identity of the genes commonly upregulated in all three stress responses is provided in Table 2.

**Table 2** Percentage of common upregulated genes during cold, drought and UV-B light stress in shoots

	Time point					
	0.5 h	1.0 h	3.0 h	6.0 h	12 h	24 h
Common upregulated genes (%)	1.9	6.3	0.5	0.7	1.0	0.3
Common transcriptional regulators (%)	66.7	35.6	0.0	21.4	26.7	16.7

**Table 3** Percentage of common upregulated genes during UV-B light stress in roots and shoots

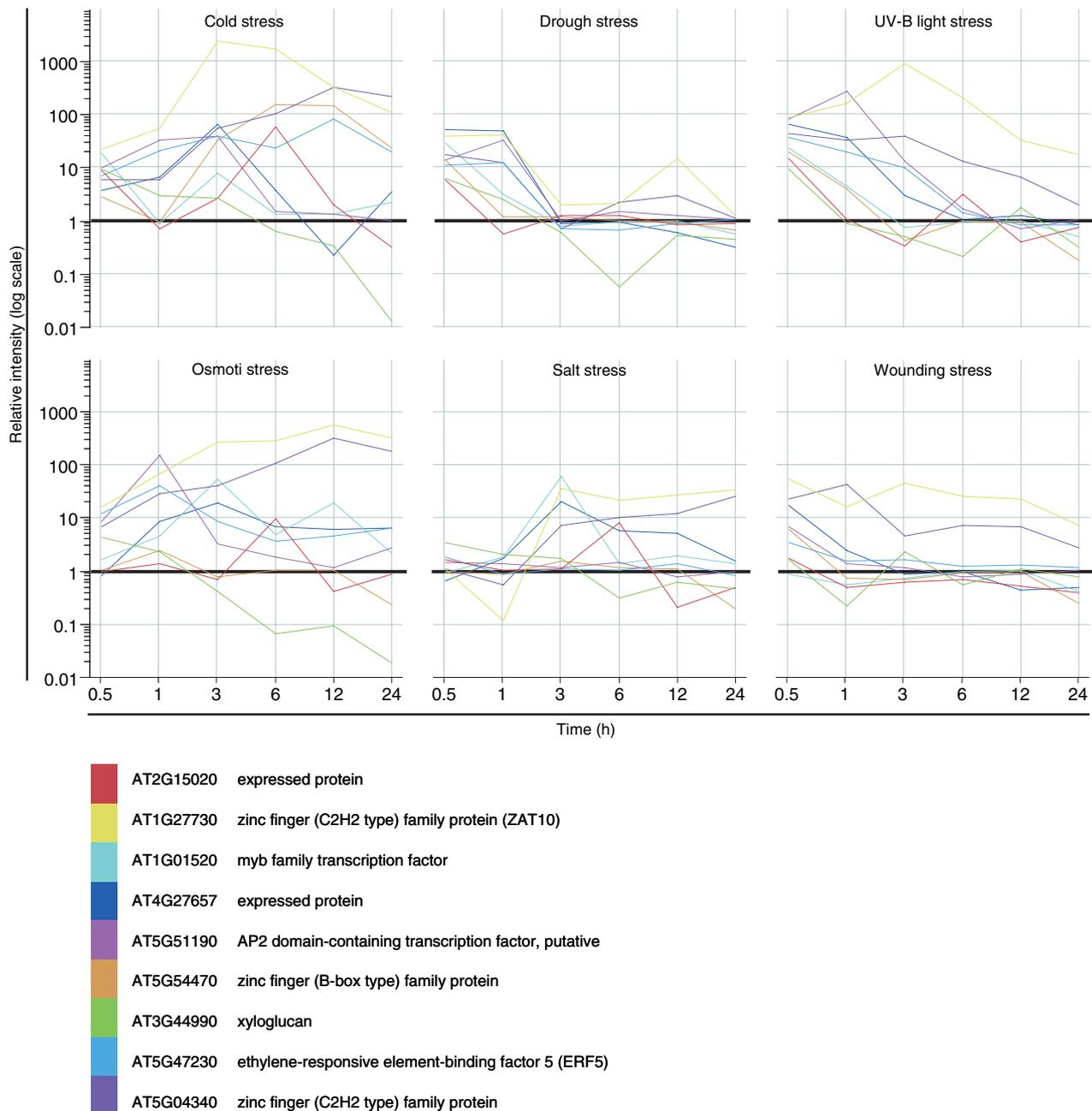
	Time points						
	0.25 h	0.5 h	1.0 h	3.0 h	6.0 h	12 h	24 h
Common upregulated genes (%)	7.8	14.4	9.8	4.1	0.6	0.6	3.4
Common downregulated genes (%)	10.5	1.1	1.5	1.7	0.0	0.0	0.0

were 15 genes for cold/UV-B light and 13 for cold/drought stress combinations.

#### *Rapidly induced genes common to drought, cold and UV-B light stress treatments*

We next concentrated our analyses on genes rapidly induced by all stresses in the shoots within the first few hours. These genes might encode plant core environmental stress response (PCESR) proteins with important functions in various stress response pathways as known for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

(Causton *et al.*, 2001; Chen *et al.*, 2003). Thirty minutes after the onset of stress treatments there was a group of nine genes induced in the whole shoot by all three stresses (Figure 3, Supplementary Table S2a). The majority of these genes were also immediately responsive to salt and osmotic stress and wounding (Figure 4). At least six of the nine fast-induced genes encode bona fide transcriptional regulators [Figure 4, Supplementary Table S2a; Arabidopsis transcription factor data base (DATF; <http://datf.cbi.pku.edu.cn>)]. However, these genes showed differential expression kinetics depending on the applied stress (Figure 4). For instance, the C2H2 zinc finger transcription factor ZAT10



**Figure 4.** Overall expression kinetics of the nine genes commonly upregulated in the shoot at the 0.5 h time point after onset of cold, drought, UV-B, salt, osmotic stress treatment and wounding. The identity of the genes is provided at the bottom.

displayed a one-peak, transient expression pattern during UV-B and cold stress, a biphasic profile during drought stress and a sustained expression during wounding (Figure 4).

Within 1 h the number of commonly induced genes increased to 59 (Figure 3). Of these 59 genes, 21 represent transcriptional regulators. In addition to members of the ERF and MYB family these again include several C2H2 zinc finger transcription factors such as AZF2, ZAT10 and ZAT12 (Supplementary Table S2b). This set of early responding

transcription factors was complemented by genes for signalling components such as, for instance, proteins involved in  $\text{Ca}^{2+}$  signalling (Supplementary Table S2b). At later time points the percentage of commonly regulated genes and the contribution of transcriptional regulators decreased significantly (Table 3, Supplementary Table S2c–f) suggesting that stress-specific response pathways had been initiated and were proceeding.

We used vector analysis (VA; Breitling *et al.*, 2005) as a means to quantitatively represent the expression dynamics

of a single gene under different experimental conditions. Here VA provides an impression of how a given gene might be controlled in the plant during simultaneous exposure to different stresses by theoretical means. We focused on the 59 genes which were concurrently upregulated in the shoot 1 h after induction during cold, drought and UV-B light stress treatment (Figure 3; Supplementary Table S2b). Because VA enables a two-dimensional representation only, we compared the stresses in pairs (Figure 5). A significant number of the 59 genes were already upregulated 0.5 h after the application of all three stresses (Figure 5a–c). The comparison of cold and UV-B light stress indicated that, at early time points, UV-B light stress slightly dominated over the regulatory influence of cold stress (Figure 5a). At later time points cold was predominant over UV-B light (Figure 4a). At the 12-h time point cold repressed the expression of some genes which were strongly upregulated by UV-B light (and cold) at 1 h. When UV-B light and drought stress were compared, it was recognizable that UV-B dominated over the regulatory influence of drought (Figure 5b). Although drought was slightly predominant over cold at 0.5 h after stress application, the latter had a stronger influence on the expression of the selected genes at later time points (Figure 5c).

#### *Principal component analysis of stress-induced gene expression*

To reveal potential response pathways specific to drought, cold and UV-B light stress, we applied a principal component analysis (PCA; Schoelkopf *et al.*, 1998) using the root and shoot data set. Principal component analysis converts large microarray data sets into a few representative numbers for each sample giving a transcriptional 'signature' for each experimental condition. These numbers can be interpreted as a measure of distance between the samples, sacrificing the information on specific genes for a global view. Thus, the closer two samples are in the PCA, the closer the similarity of the overall transcriptional expression.

At the very early time points after onset of stress application the samples clustered together indicating a high similarity in the early transcriptional responses to the different stresses (Figure 6). This suggests that the immediate response of the plant to these stresses is non-specific and contains only minor tissue-specific elements. However, at later time points stress-, tissue- and time-specific responses became apparent. For instance, UV-B light irradiation of the shoots induced a large and distinct transcriptional response which differed significantly from that caused by the other stress treatments (Figure 6). Furthermore, although the roots of UV-B-treated plants clearly responded to the shoot-applied stress, the reaction is not as intense and UV-B light-specific as in the shoot (Figure 5). In contrast, the shoot and root tissue showed a very similar transcriptional

response to cold stress, although the response was delayed in the root (Figure 5). Similar results were obtained when all other abiotic stresses were included in the PCA (Supplementary Figure S4).

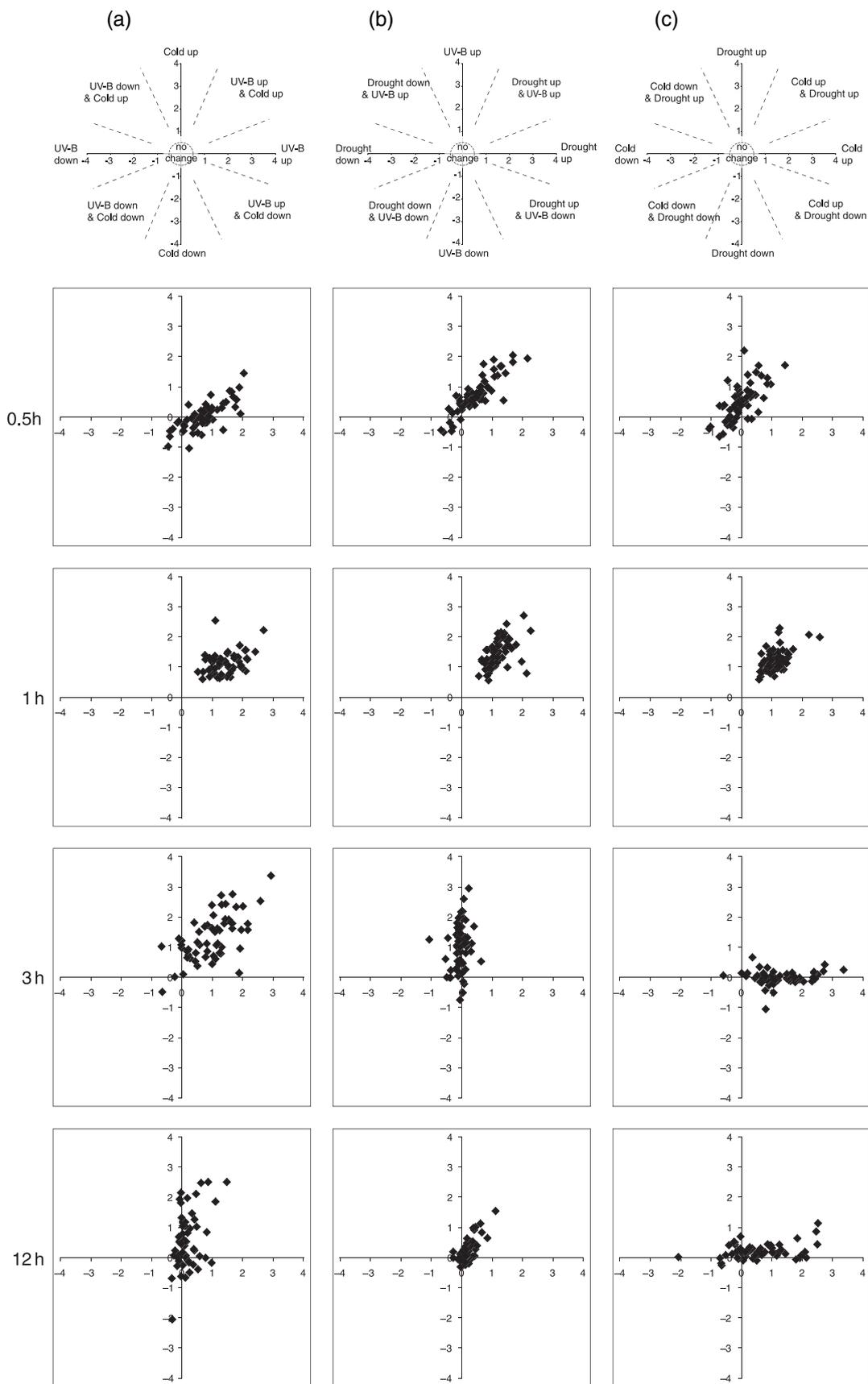
#### *Global changes in gene expression in response to UV-B light*

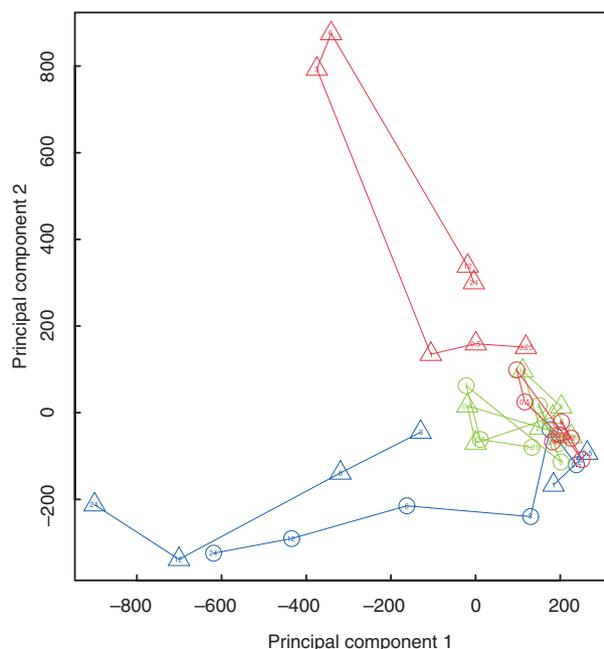
We next performed a more detailed investigation of spatio-temporal changes in the number of differentially expressed genes during the UV-B light-induced response. The UV-B light stress was of special interest because it produced a very fast and transient gene expression response and included a systemic shoot-to-root signalling aspect (Figure 2).

This analysis revealed that 100 genes in the shoot and 39 genes in the root displayed a strong upregulation 15 min after the onset of UV-B light irradiation (Figure 7). Fifteen minutes later this number increased to around 300 in the shoot and 200 in the root. This increase continued until a maximum was reached after 6 h in the shoot and 3 h in the root (Figure 2, Table 1). Afterwards we observed a sharp drop of differentially regulated genes in both tissues. A similar pattern was recognizable for genes downregulated by UV-B (Figure 2).

A high proportion (18%; representation in the genome 12.4%) of genes upregulated in shoots within 30 min of the onset of irradiation represent transcriptional regulators of various families. Among these we identified members of the B-box-, CCCH-, C2H2- and C3HC4-type zinc finger proteins, WRKY transcription factors, transcriptional regulators involved in the ethylene response pathway (ERFs) and those participating in auxin signalling (Supplementary Table S3). In addition, genes likely to encode signalling components, as for example different types of kinases and proteins involved in Ca<sup>2+</sup> signal transduction, were identified among the immediate response genes (Supplementary Table S3). Notably, although the plants were grown under sterile conditions and had not been exposed to any pathogen, a large number of genes annotated to be involved in pathogen signal transduction and defence response as well as in oxidative stress signalling and adaptation were upregulated by irradiation with UV-B light (Supplementary Table S3). Whereas the proportion of genes coding for transcriptional regulators, signalling elements and pathogen-related proteins stayed high over the entire time period investigated, the genes representing components of certain metabolic pathways (e.g. flavonoid biosynthesis), elements of the chaperone/protein degradation machinery and various transporters were predominantly induced at later time points (1–6 h after onset of irradiation) in both roots and shoots (data not shown).

The general expression patterns presented in Figure 6 indicate that Arabidopsis responds to a 15-min pulse of UV-B light by a transient induction of thousands of genes. However, a detailed view of the kinetics revealed that this





**Figure 6.** Principal component analysis of the cold (blue) drought (green) and UV-B light (red) data sets.

Principal component analysis was applied to 40 stress treatment samples exhibiting clear differences for specific stresses, tissues, and time points. The 40 stress samples included root (circles) and shoot (triangles) tissue for the indicated time points (see Experimental procedures).

pattern comprised, on the one hand, expression transients occurring within a short period of minutes or a few hours and, on the other, sustained expression over a long period of several hours (Figures 7 and 8). For instance, of the 100 genes induced in the shoot after 15-min UV-B light, 26 were no longer upregulated 15 min later (Figure 7b). However, the transcripts of some of these genes reappeared a few hours later indicating a second transient of UV-B light-induced gene expression (Figure 8). A similar expression profile was observed in the root (data not shown). It is noteworthy that the major proportion (30.8%) of these 26 transiently expressed genes encode kinases, Aux/IAA proteins and, particularly, components involved in pathogen defence and, to a minor extent, transcriptional regulators (Supplementary Table S3). This transient expression principle was also observed when later time points were included in the analysis. Of the 535 genes upregulated in the shoot 1 h after UV-B light treatment only 67 were already induced at 15 min and 211 at 30 min, respectively (data not shown).

Thus, a large proportion of UV-B light-regulated gene expression, which is very likely to be responsible for the initiation of biochemical, physiological and morphological adaptation responses, appears to happen in the first response phase of 30 min and in a second response phase several hours later.

Due to the design of the growth boxes, UV-B light was exclusively absorbed by the aerial parts of the irradiated plants. The concurrent harvest of shoots and roots from identically treated plants offered us the unique possibility of determining the extent to which UV-B irradiation regulates common or different genes in the shoot and the root – an approach which was not previously possible (Brown *et al.*, 2005; Ulm *et al.*, 2004). At early time points (15 to 60 min after the onset of irradiation) only around 10% of genes upregulated by UV-B light were identical in the shoot and the root tissue (Table 3, Supplementary Table S4). At later time points the proportion of identical genes declined even more. The low percentage of commonly expressed genes as well as the downward tendency over time was more pronounced in the case of UV-B downregulated genes (Table 3). In the initial phase (15 min after the onset of irradiation) around 10% of UV-B light-repressed genes were identical in both tissues. This proportion decreased continuously at later time points (Table 3).

The genes upregulated in common in the roots and the shoots within the first hour after UV-B light exposure encode transcriptional regulators of various families (19.8%), signalling elements and components participating in pathogen signal transduction or defence (Supplementary Table S4). The upregulation of *long hypocotyl in far-red* (*HFR1*) and *long hypocotyl 5* (*HY5*) suggests that components of the UV-B stress response pathway and elements required for the photomorphogenic UV-B light response pathway (Ulm and Nagy, 2005) were induced in both roots and shoots. Furthermore, we observed a fast and transient upregulation of the *PHR1* gene, of which the gene product, a CPD photolyase, plays a major role in the repair of UV-B light-induced DNA damage.

The overall overlap of our UV-B light stress data with the data set published by Ulm *et al.* (2004) is shown in Supplementary Figure 5a. Whereas Ulm *et al.* observed 660 (1716) genes differentially regulated 1 h (6 h) after onset of the irradiation, 758 (1274) genes responded in this study. There was overlap of 383 (1 h) and 816 (6 h) genes found in both studies, unravelling a robust set of UV-B light-inducible genes, although – with the exception of the UV-B light

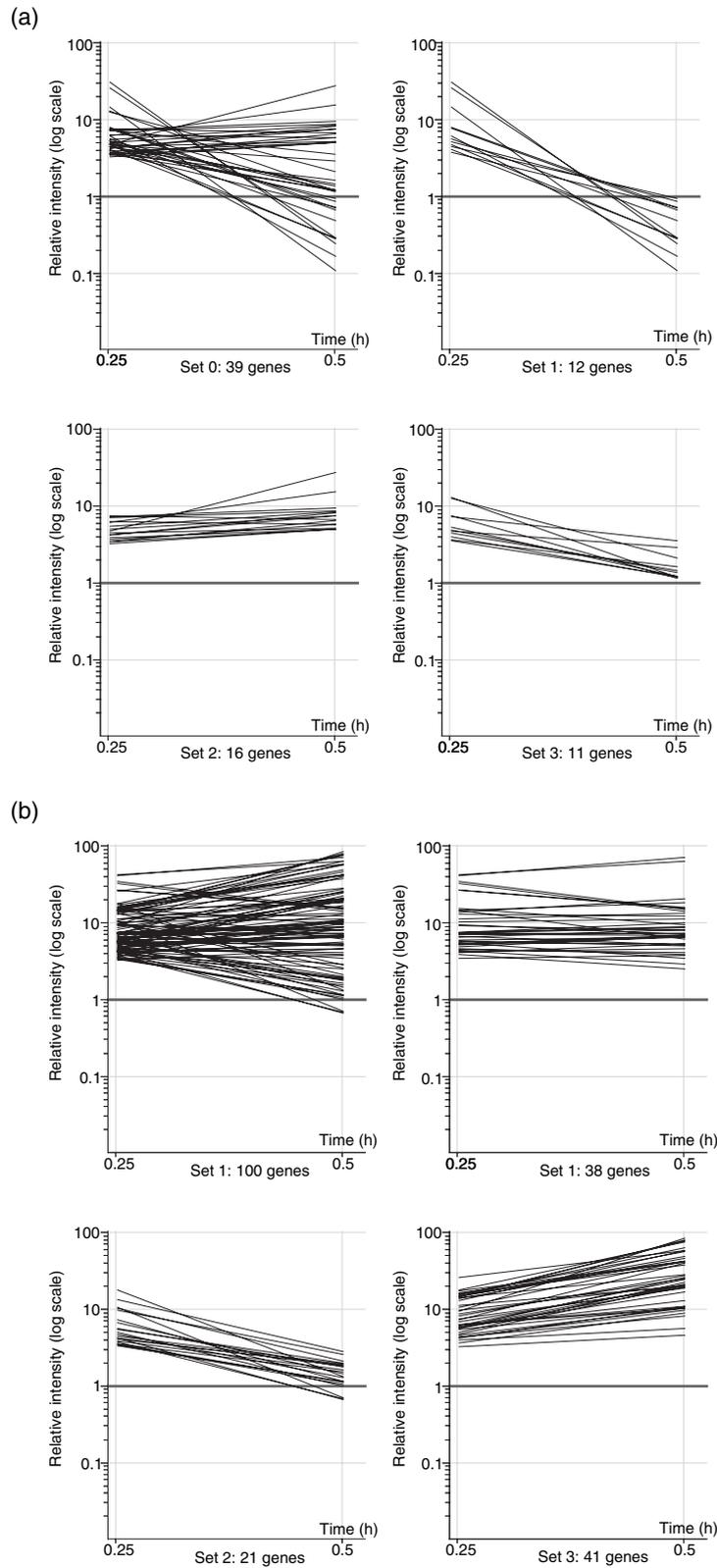
**Figure 5.** Vector analysis (VA) of genes commonly upregulated in the shoot by cold, drought and UV-B light stress.

The VA over time (0.5 to 12 h) was carried using the 59 genes (squares) commonly upregulated in the shoot 1 h after onset of the stress treatment according to Breitling *et al.* (2005). For details see Experimental procedures.

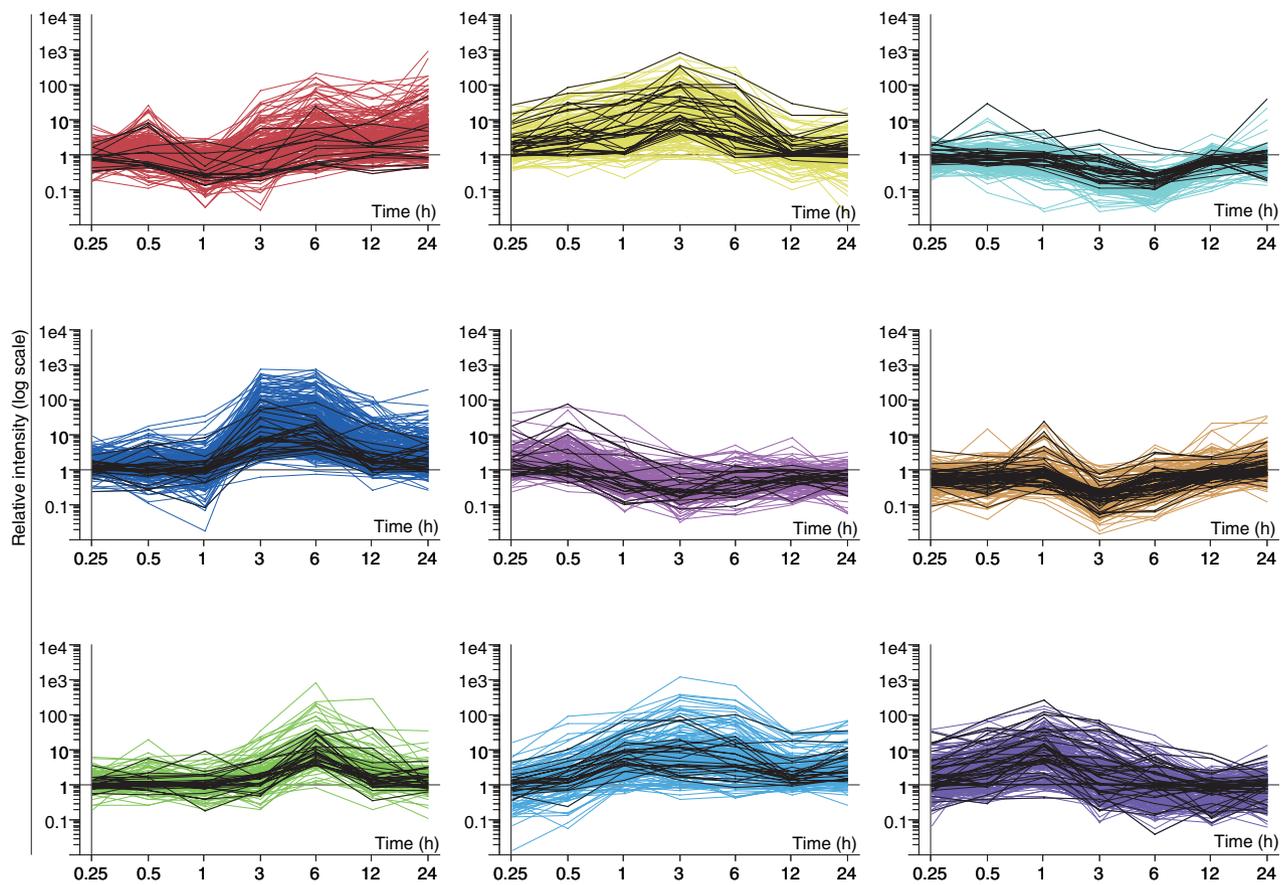
(a) Vector analysis of cold versus UV-B light stress.

(b) Vector analysis of UV-B light versus drought.

(c) Vector analysis of drought versus cold.



**Figure 7.** Expression profiles of genes immediately upregulated in the root and the shoot after 15 min in response to irradiation with a 15-min UV-B light pulse. Time course of the relative expression levels of the genes differentially regulated between 0.25 and 0.5 h after the onset of UV-B light irradiation in the shoot (a) and the root (b). The upper left diagram in (a) and (b) shows the complete set of genes, whilst the other diagrams display the divergent expression kinetics of these genes within 30 min. The red line shows the expression level of these genes in mock-treated control plants. The identity of the genes is provided in Supplementary Table S4.



**Figure 8.** Differential expression pattern of genes upregulated by a 15-min UV-B light pulse. Kinetic clusters of gene expression pattern in the shoot induced by a 15-min UV-B light pulse. All upregulated genes could be included in one of the kinetic clusters. The black lines show representative transcriptional regulators. Clustering was performed as described in Experimental procedures.

source and intensity used – the experimental set-up was different. Furthermore, with one exception, the 39 genes responsive to UV-B light found by Brown *et al.* (2005) to be transcriptionally dependent on the activity of *UVR8* and *HY5* were also found in our data set (Supplementary Figure S5b). However, the vast majority of the genes differentially regulated by damaging UV-B light under our conditions were not identified by Brown *et al.* (2005) suggesting that they are transcriptionally independent of the *UVR8* and *HY5* gene products.

## Discussion

In the present work we describe the protocol for the generation of highly reproducible and organ-specific microarray data sets of various stress responses in *Arabidopsis thaliana*, which were generated in the framework of the DFG-funded AtGenExpress project. The high quality, comparability and reliability of the abiotic stress data sets enable in-depth correlative data analyses of the transcriptional stress response in *Arabidopsis*. Recent examples

have been published or are going to be published on the dissection of the salt stress pathway in *Arabidopsis* (Bohnert *et al.*, 2006; Ma *et al.*, 2006), the development of novel clustering approaches over multiple time courses and between the described abiotic stresses (Bleuler and Zitzler, 2005; M. Strauch, J. Supper, C. Spieth, D. Wanke, J. Kilian, K. Harter, A. Zell, unpublished data; J. Supper, M. Strauch, D. Wanke, K. Harter, A. Zell, unpublished data; Swindell, 2006) and the identification of *cis*-regulatory elements involved in abiotic stress-controlled gene expression (Berendzen *et al.*, 2006; Walther *et al.*, 2007; M. Strauch, D. Wanke, J. Supper, J. Kilian, K. Harter, A. Zell, K. Berendzen, University of Tübingen, Tübingen, Germany, unpublished data). On the one hand, this is a consequence of the side-by-side growth of genetically homogeneous plant material, the coincident application of the stresses and the identical tissue harvesting and RNA extraction protocols. On the other hand, we included early time points (0.25 h and/or 0.5 h) after the onset of stress treatment in our analyses to identify immediate stress responsive genes. In summary, our stress data set

provides a unique experimental basis for the development of novel bioinformatics tools for the three-dimensional analysis of gene clusters, the identification of functionally related *cis*-acting elements, the time-resolved determination of the activity of stress-modulated gene regulatory networks and other bioinformatics and experimental approaches.

#### *Global gene expression pattern in response to abiotic stresses*

Our initial analysis determined the global spatio-temporal gene expression pattern of *Arabidopsis* in response to heat, cold, drought, salt, osmotic, wounding and UV-B light stress. Furthermore, we performed a PCA (Schoelkopf *et al.*, 1998) to unravel global stress and tissue-specific patterns.

In general, abiotic stress-modulated alterations in gene expression occur fast. The first changes are observed within 30 min of the application of stress. Immediate changes are detected in both tissues independent of whether the whole plant or only the shoot or the roots were exposed. The latter observation suggests the immediate production of a systemic signal by the shoot or root which is transferred to the other organ.

The total number of differentially expressed genes and the pattern of how these genes are up- or downregulated over time differs distinctly between the different stimuli. A very striking example is the different gene expression pattern induced by the continuous application of salt or osmotic stress. As indicated by the transient expression pattern, the plant is able to cope with the high salt conditions. In contrast, high osmolarity induces a continuous response which dramatically increases with time. This observation implies that the plant clearly differentiates between the ionic and the osmotic contribution of stresses at a global gene expression level.

In spite of these differences, there is a set of common genes that we found to be rapidly induced independent of the applied abiotic stress. Around 50% of these common immediately responsive genes represent transcriptional regulators which might encode basic, but non-specific, master regulators generally required for the PCESR. These early-induced, common genes are not related to those responsible for the CESR of yeast and fission yeast (Causton *et al.*, 2001; Chen *et al.*, 2003), suggesting that plants have evolved a distinct early stress response.

Our VA provides an initial idea of how the complex dynamics of gene expression might be established in the plant, when several abiotic stresses act on *Arabidopsis* in parallel. However, how the plant actually integrates the stress input information over time at gene expression level, and whether this integration can be predicted and modelled by using bioinformatics tools such as VA, must be deter-

mined in future after performing multi-stress factor microarray experiments.

An interesting finding of our PCA and other analyses is that a specific expression response is not established in the plant immediately after the onset of stress treatment. Stress-specific output reactions at the gene expression level first become detectable 1 to 3 h later. Obviously, the very early response of the plant to abiotic stress is rather non-specific. This might point to common initial signalling events. For drought, cold, UV light and other abiotic stresses the production of ROS (Apel and Hirt, 2004; Mahalingam and Fedoroff, 2003) has been reported. Besides  $\text{Ca}^{2+}$  changes, the generation of ROS is one crucial event known so far to be common among such divergent stresses and may function in integrating the responses of plants to abiotic and biotic stresses (Kuzniak and Urbanek, 2000; Mittler and Zilinskas, 2004; Rodriguez and Redman, 2005). It is, therefore, reasonable to assume that ROS may serve as an important initial signal for the immediate abiotic stress reaction which includes the regulation of early master genes common to all stresses. This is in agreement with our finding that the C2H2-type zinc finger transcriptional regulators AZF2, ZAT10 and ZAT12 were identified in the group of rapidly and generally stress-induced genes. ZAT12 has recently been suggested to play a central role in reactive oxygen and abiotic stress signalling in *Arabidopsis* (Davletova *et al.*, 2005). It is noteworthy that these zinc finger proteins probably act as transcriptional repressors on carbohydrate metabolism and photosynthesis under conditions of abiotic stress. This immediate onset of a metabolic reprogramming may enable the adjustment of energy homeostasis to the stress conditions which is necessary for a successful stress defence (Ohta *et al.*, 2001; Sakamoto *et al.*, 2004).

When the PECSR proteins mainly adjust the energy balance, the realization of specific stress responses may require additional input information which is unique to the applied stress (e.g. physical properties, dose and duration of the stress, tissue-specific elements). This is translated into the expression of stress-specific genes in a defined spatio-temporal manner. The combinatorial action of immediate-responsive general repressors and spatio-temporally upregulated, specific elements may eventually lead to the initiation of a regulatory gene expression network resulting in a specific physiological readout.

#### *The stress response to UV-B light*

Our detailed analysis of the global gene expression pattern after irradiation with UV-B light revealed that the plant is able to respond within 15 min with the expression of around 100 genes which mainly encode transcriptional regulators, signalling elements and pathogen-related proteins. A major proportion of these early induced genes show a biphasic transient appearance with a primary peak at around 1 h after

irradiation, followed by a secondary peak of similar magnitude some hours later. The fact that the early induced genes encode pathogen-related proteins and the biphasic kinetics of the expression suggest that UV-B light induces an oxidative burst in plant cells similar to that of pathogens (Mahalingam and Fedoroff, 2003).

When the complete UV-B light data set was included in the analysis, it became obvious that UV-B light caused a complex kinetic pattern of gene expression transients in both shoots and roots. Again, the magnitude and diversity of the expression response is similar to what has been documented as a systemic reaction after pathogen attack (Kunkel and Brooks, 2002). Whether these transients are causally linked to one another (e.g. an early wave of transcriptional regulators induces a later wave of gene expression) or are independently initiated by different signalling events remains to be elucidated. The *in silico* discovery of potential functionally related *cis*-regulatory elements using novel bioinformatics tools (Berendzen *et al.*, 2006; Walther *et al.*, 2007) in combination with high-resolution co-expression analyses may help to solve this challenging task.

The irradiation of the shoot with UV-B light also induced an immediate response in the gene expression of the non-irradiated root. This observation suggests the production of a systemic signal by the shoot which is subsequently transferred extremely fast to the root. This signal does not appear to carry UV-B-specific information because, according to the PCA, the stress-induced expression profile of the root is not stress specific and is very different from that of the shoot. Thus, the shoot 'informs' the root about the perception of a stress signal without specifying its exact nature. In contrast, when the plants were exposed to cold stress, the shoot and the root displayed a high percentage of co-expression of identical genes. This demonstrates that the root is in principle able to respond in a stress-specific way.

A long-distance systemic signal was also proposed for the UV-B light-induced transcriptome response in maize. Corresponding to our observation in Arabidopsis, the irradiation of aerial maize tissues with UV-B light triggered a rapid expression response not only in shielded leaves but also in roots and immature ears (Casati and Walbot, 2004). However, the nature of the signal(s) produced in irradiated cells, which elicits rapid transcriptome changes in distant shielded tissues and organs, remains to be elucidated.

## Conclusion

Here we describe the detailed protocols for the cultivation, stress exposure and sample harvesting of the plants which were used for the generation of the AtGenExpress microarray abiotic stress data set. These data are already in intensive use by scientists. In addition, they proved to be an important reference to the Arabidopsis stress-regulated transcriptome and a principal information resource for stress data mining

by researchers worldwide. Coupled with detailed quantitative and physiological kinetics analyses, this kind of combinatorial study will move us closer to answering the question of how plants cope with environmental stress.

## Experimental procedures

### Standard growth conditions

Plants were grown under sterile conditions at 50% relative humidity, 24°C and a light regime of 16 h light and 8 h dark in a phytochamber. For cultivation we used growth boxes containing rafts (LifeRaftR) supported by floats (Raft Float) in growth boxes (LifeGuardR), which were closed with a membrane vented lid. This growth equipment was obtained from Osmotek (<http://www.osmotek.com/>). The white light source consisted of 50% Osram 36W/21-840 Lumiluse Plus Cool and 50% Osram L58W/77 Fluora tubes (<http://www.osram.de>) and generated a light intensity of 150  $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$ .

Before sowing, seeds of *A. thaliana* ecotype Col-0 were shaken for 10 min in 70% ethanol. The ethanol was replaced by 25% (v/v) dilution of 12% sodium hypochlorite solution (bleach) and incubated for an additional 10 min. Subsequently, the seeds were washed five times with sterile water. Nine seeds were placed on each raft with toothpicks, and the rafts were transferred into the growth boxes containing MS/agar initial cultivation media [50 ml of 0.5 $\times$  MS supplemented with 1 $\times$  Gamborg B5 vitamins, 0.5% (w/v) sucrose, 0.5% (w/v) agar, pH 5.7]. For stratification, the boxes were incubated for 2 days at 4°C in darkness. Thirteen days after sowing, the rafts were transferred into new growth boxes containing 75 ml of MS media without sucrose and agar (for composition see above).

### Tissue harvest, RNA isolation and DNA chip hybridization

Approximately 100 mg shoot and root tissue was harvested separately, transferred into 2 ml reaction tubes containing a 3 mm tungsten-carbide bead (Qiagen, <http://www.qiagen.com/>) and ground to a fine powder using a mixer mill M300 (Retsch Mixer Mill system, <http://www.retsch.com/>). Extraction and purification of total RNA was carried out according to the RNeasy Plant Mini system using the buffers provided (Qiagen) with the following modifications: After adding 450  $\mu\text{l}$  (shoot tissue) or 900  $\mu\text{l}$  (root tissue) of extraction buffer, two cycles of heating (1 min, 56°C) and freezing in liquid nitrogen were performed. The samples were then treated according to the RNeasy mini protocol (chapter 'Plant and fungi'). Ribonucleic acid was eluted from the columns twice with 50  $\mu\text{l}$  of 62°C heated RNase-free water. Purified total RNA was precipitated and resuspended in water to a final concentration of at least 1  $\mu\text{g } \mu\text{l}^{-1}$ . A detailed protocol has been deposited at TAIR (<ftp://ftp.arabidopsis.org/home/tair/Protocols/AGE-Probe-Isolation.pdf>). Further processing of the RNA and cRNA hybridization using the Affymetrix AHT1 gene chip was accomplished according to a modified Affymetrix protocol developed at the Deutsche Ressourcenzentrum für Genomforschung (RZPD) in Berlin, Germany (<http://www.rzpd.de/>).

### Microarray, raw data, VA, PCA and functional categorization

For expression analysis the Affymetrix CEL files generated at the RZPD, Berlin, containing the raw probe intensity values from 224 ATH1 gene arrays [accession numbers at TAIR (<http://www.arabidopsis.org>) are: cold stress, ME00325; drought stress, ME00338; UV-B light stress, ME00329; salt stress, ME00328; osmotic stress,

ME00327; wounding, ME00330; heat stress, ME00339] were imported into GeneSpring software version 7 (Agilent, <http://www.agilent.com/>). A per-chip normalization to the median was applied to obtain comparability. The arrays were adjusted for background of optical noise using the GC-RMA software (Wu *et al.*, 2004) and normalized using quantile normalization. The quality of the replicates was tested by performing a least-square regression analysis (Supplementary Figure S2). From the resulting signal intensities, fold change values were calculated. A gene was classified as upregulated when the signal intensities for both treatment replicates were at least threefold higher than the signal intensities for both control replicates. Similarly, a gene was classified as downregulated when the intensities for both control replicates were at least threefold higher than those of both treatment replicates. By comparing each duplicated control array with its partner for every time point, it was found that a fold change cut-off of  $\geq 3$  results in an average of 0.4% of genes falsely classified as up- or downregulated. By comparing two treatments against two controls, the false discovery rate was estimated to be  $< 3 \times 10^{-10}$ . Table and chart management was performed with Microsoft Excel. To determine overlaps between the differentially regulated genes, and for visualization, the Venn diagram option was used. The genes were categorized into functional groups using the Functional Catalogue at MIPS (<http://mips.gsf.de>) with manual adjustment when necessary.

Vector analysis was performed with the perl script according to Breiting *et al.* (2005). For the fold change input log ratios ( $\log_{10}$  of control versus treated) for every combination was used (for one condition/stress four combinations). The procedure was carried out for every combination of the stress cold, drought and UV-B stress shoot data for the time points 30 min, 1 h, 3 h and 12 h.

Principal component analysis was performed using a kernel-PCA algorithm (Schoelkopf *et al.*, 1998), reducing the 22 746 gene expression values to three dimensions. This was implemented using the kernlab package (Karatzoglou *et al.*, 2004) with a linear kernel function and default settings.

To show the characteristic of the kinetics of regulated genes a clustering was performed using the *k*-means tool in GeneSpring. Pearson correlation was used as a similarity measure. Five random starting clusters of the respective gene list were tested. The number of iterations was set to 100. The data were then normalized to the control data. The following formula was applied to all time points:

$$\text{relative intensity} = \frac{\text{signal strength of gene A in sample X}}{\text{average signal strength of gene A in control samples}}$$

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## Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Cooling diagram of the cold stress experiment. Decrease of the temperature in the culture medium after transfer of the

growth boxes onto ice in the cold room. A temperature of 3°C is reached after around 30 min.

**Figure S2.** Replicate quality of the biological duplicates of the array. The  $r^2$  linear regression value was computed for all array duplicates, indicating similarity between the replicates (a value of 1 means a perfect correlation between gene expression measures). The correlation between the replicates was never less than 0.95. Values of 3 h UV-B light (root) show the highest  $r^2$  value while those of 3 h cold (root) show the lowest. Similar results documenting the high correlation between the data points were also obtained for the other abiotic stress data set replica (data not shown). The values were computed using the R statistics package (<http://www.r-project.org/>).

**Figure S3.** Expression profiles of selected abiotic stress marker genes induced by drought. (a) This study: Profile after 15 min incubation of the plants in an air stream until 10% loss of fresh weight was reached. (b) Seki *et al.* (2002): Profile during continuous drought stress by placing the plants, which have been removed from the substrate (agar), on a plastic dish at 22°C.

**Figure S4.** Principle component analysis (PCA) of all abiotic stress data sets. PCA was applied to 92 stress treatment samples exhibiting clear differences for specific stresses, tissues and time points. The stress samples include root (circles) and shoot (triangles) samples for a time course for the indicated six stresses. For further details see Experimental procedures.

**Figure S5.** Overlap of the genes up-regulated in response to UV-B light stress by comparing the results of this study and those of the study by (a) Ulm *et al.* (2004) and (b) by Brown *et al.* (2005). (a) Genes up-regulated in response to UV-B light 1 h (left) and 6 h (right) after irradiation. The total number of genes in the root and the shoot found in this study are shown in red, whereas those found in the study by Ulm *et al.* (2004) are shown in green. (b) Total number of UV-B up-regulated genes found in this study (red) compared to those (green) which are dependent on HY5 and UVR8 (Brown *et al.*, 2005).

**Table S1** Comparison of material, methods and experimental procedures from selected publications related to global analyses of abiotic stress-induced gene expression

**Table S2** Common upregulated genes in cold, drought and UV-B light stress in the shoots 6 h after onset of treatment

**Table S3** Up-regulated genes in the shoots 0.25 h to 0.5 h after onset of the UV-B light treatment Genes up-regulated 0.25 and 0.5 h after onset of stress treatment

**Table S4** Genes regulated by UV-B light stress in roots and shoots This material is available as part of the online article from <http://www.blackwell-synergy.com>

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