Supplementary Methods

Affymetrix GeneChip sample preparation

12 µl total RNA and 1 µl MluT7-oligo-dT primer

(5´-GGAGGCCGGAGAATTGTAATACGACTCACTATAGGGAGACGCGTGT$_{20}$-3´) were mixed in a PCR tube, heated to 70°C for 3 min, and cooled on ice. 4 µl 5x RT buffer, 1 µl 100 mM DTT, 1 µl 10 mM dNTPs and 1 µl Superscript III reverse transcriptase (SSIII, Invitrogen GmbH, Haus 223, A-5090 Lofer, Austria) were added, mixed and icubated for 30 min at 50°C and 30 min at min 55°C. SSIII was deactivated by incubating for 15 min at 70°C.

To the first strand cDNA were added 91 µl water, 30 µl 5x Second Strand Buffer, 3 µl 10 mM dNTPs (Roche Deutschland Holding GmbH, Emil-Barell-Str. 1, D-79639 Grenzach-Wyhlen, Germany), 1 µl *E.coli* DNA Ligase (Roche), 4 µl DNA Polymerase I (Roche), and 1 µl RNase H (Roche). This mixture was incubated for 2 h at 16°C in a PCRcycler (without heated lid). The enzymes were deactivated for 10 min at 75°C.

The second strand cDNA was purified with a QIAquick PCR purification kit (Qiagen) essentially as described by the manufacturer and the column was eluted with 50 µl 1 mM TRIS/HCl pH8. The cDNA was transcribed using the Ambion MEGAscript T7 Kit (Ambion, Inc., 2130 Woodward, Austin, TX 78744-1832, USA) as follows: The volume was reduced to 2 µl and 6 µl water, 2 µl ATP solution, 2 µl CTP solution, 2 µl GTP solution, 2 µl TTP solution, 2 µl 10x Reaction Buffer and 2 µl Enzyme Mix were added at room temperature, mixed and incubated 5 h at 37°C. The amplified RNA (aRNA) was purified with a RNeasy MinElute column (Qiagen) and eluted with 13 µl water.

11.5 µl T7 aRNA in water was mixed with 1.5 µl N6 primer and heated to 70°C for 3 min. The tube was cooled on ice, centrifuged, and 4 µl 5x RT buffer, 1 µl 100 mM DTT, 1 µl 10 mM dNTPs, and 1 µl SSIII were added. After mixing the tube was incubated for 5 min at 25°C, 5 min at 37°C, and 50 min at 50°C. SSIII was deactivated by incubating for 15 min at 70°C.

To the 20 µl first strand cDNA 1 µl RNaseH was added and incubated for 20 min at 37°C and 1 min at 94°C. The reaction was cooled on ice and centrifuged. 1 µl MluT7-oligo-dT primer was added and heated to 70°C for 3 min, then put on ice. After centrifugation 64 µl water, 10 µl 10x Orange Buffer (Fermentas), 3 µl 10 mM dNTPs, and 1 µl Klenow Enzyme (Roche) were added. After mixing the tube was incubated for 1 h at 37°C followed by 10 min at 75°C. and put on ice. The cycle 2 second strand cDNA was purified as described for cycle 1 second strand cDNA. The purified cDNA was either used for another amplification round (cycle 3) or transcribed and labeled as described below.

cRNA was produced and labeled by T7 RNA *in vitro* transcription using the Ambion MEGAscript T7 Kit. The cDNA from either cycle 2 or cycle 3 was reduced to 2 µl. 2 µl ATP solution, 1.5 µl CTP solution, 2 µl GTP solution, 1.5 µl UTP solution, 3.75 µl Bio-11-CTP solution (NEN), 3.75 µl Bio-11-UTP solution (NEN), 2 µl 10x Reaction Buffer, and 2 µl Enzyme Mix were added at room temperature and incubated for 5 h at 37°C. The labelled RNA was purified with a Qiagen RNeasy column as described by the manufacturer and eluted with 2x 30 µl water. A Bioanalyzer run was used to check the quality of the transcribed RNA and to determine the amount of RNA.

Fragmentation of cRNA and preparation of the hybridization mix containing fragmented, biotinylated cRNA at a concentration of 60 ng/µl were carried out according to the *GeneChip® Expression Analysis Technical Manual* (Affymetrix, Santa Clara, USA). ATH1 arrays (GeneChip Arabidopsis ATH1 Genome Array; Affymetrix, Santa Clara, CA, USA) were hybridized for 16 to 18 hours at 45°C and 60 rounds per minute in a hybridization oven (*Hybridization Oven 640*, Affymetrix, Santa Clara, USA). Washing of arrays and staining with streptavidine-phycoerythrine was performed in an automatic fluidics station (*GeneChip Fluidics Station 400*, Affymetrix, Santa Clara, CA, USA) using the *EukGe_WS2v4* protocol. Scanning of arrays was performed with the *GeneChip Scanner 3000* (Affymetrix, Santa Clara, CA, USA).

Statistical analysis of microarray data

Affymetrix CEL files were read into the R statistical analysis environment (www.r-project.org) using the *affy* package of the Bioconductor suite (www.bioconductor.org). As 10–40% of probe sets are affected by updated gene annotation, chips were processed with current TAIR v8 probe-set annotation (Dai, M. et al. 2005). Probe sequence specific 'background correction' (Wu, Z. et al. 2004) was performed using routines available in the Bioconductor *gcrma* package. Using the 'affinity' model, while 'MM' probes were employed for the determination of affinity parameters, only 'PM' probes were used for the probe-specific background correction. An inspection of exploratory pairwise scatter and 'MA' plots confirmed the need for inter-chip normalization. The thus required explicit normalization steps made a subtraction of the heuristic estimate for optical instrument background as offered in gcrma unnecessary. Defaults were used for all other *gcrma* parameters. As an examination of pairwise quantile-quantile plots showed only random fluctuations, inter-chip normalization could be achieved using quantile-quantile normalization (Bolstad, B. M. et al. 2003). See 'Low-level microarray analysis and diagnostic plots' section of the Online Supplement for diagnostic plots and figures.
After normalization, robust summaries of probe set signals were obtained for each gene using an iterative weighted least squares fit of a linear probe level model (Bolstad, B. M. 2004) through the *fitPLM* function of the Bioconductor package *affyPLM*. This process automatically identifies unreliable chip areas and correspondingly downweights outlier probes. See Online Supplement for figures.

The normalized data on $\log_2$ scale were then fitted gene by gene with a linear model including hybridization batch effects, using the *lmFit* function (Smyth, G. K. 2004) of the Bioconductor package *limma*. The result-tables also include *q*-values as indicators of significance of contrasts after correction for multiple testing controlling the False Discovery Rate (Benjamini, Y. and Hochberg, Y. 1995). For the statistical tests, individual gene variances have been moderated using an Empirical Bayes approach that draws strength from transferring variance characteristics from the set of all genes to the test for each individual gene (Smyth, G. K. 2004).

Full GO annotation was downloaded from TAIR on 6 Jan 2007 (www.arabidopsis.org). Annotation (including 'unknown' assignments) was available for almost all genes on the chip (99.6%, 21,053). To permit analyses of arbitrary GO categories, GO-IDs were processed resolving obsolete-IDs (www.geneontology.org, revision 1.287, 6 Jan 2007), secondary IDs/aliases (rev. 1.48, 5 Jan 2007), and annotation was revised for consistency by fully recursive propagation of category membership to parent nodes. For each category, we then tested for relative enrichment of genes in the test set by comparison to the distribution of genes on the chip by Fisher's exact test and Bonferroni correction for multiple testing of the N=4,279 examined categories. This corresponds and is equivalent to the commonly employed tests using the hypergeometric distribution. Results are provided in the 'Analysis results section' of the Online Supplement.
To further characterize the nature of regulatory changes, in this paper, we tested whether significantly regulated genes were preferentially up- or downregulated. In an assessment of overrepresentation of upregulated genes in comparison to downregulated genes, we compared the distribution across GO categories of the 3,885 annotated genes that were upregulated significantly for q<5% to that of an

equal number of most significantly downregulated genes. Similarly, examining overrepresentation of downregulated genes in comparison to upregulated genes, we compared the distribution across GO categories of the 3,331 annotated genes that were downregulated significantly for q<5% to that of an equal number of most significantly upregulated genes. P-values for a significance assessment of the observed differences from the binomial distribution were Bonferroni corrected for testing of all GO categories (N=4,279) and are also provided in the 'Analysis results section' Online Supplement. Results for selected categories are presented in the paper (Figures 2 a-b and 3).

References

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