Robin, a user-friendly application for microarray analysis

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Robin: An intuitive wizard application for R-based expression microarray quality assessment and analysis.

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ABSTRACT

The wide application of high-throughput transcriptomics using microarrays has generated a plethora of technical platforms, data repositories and sophisticated statistical analysis methods, leaving the individual scientist with the problem of choosing the appropriate approach to address a biological question. Several software applications that provide a rich environment for microarray analysis and data storage are available (e.g. GeneSpring, EMMA2), but these are mostly commercial or require an advanced informatics infrastructure. There is a need for a non-commercial, easy-to-use graphical application that aids the lab researcher to find the proper method to analyze microarray data, without this requiring expert understanding of the complex underlying statistics, or programming skills. We have developed Robin, a Java-based graphical wizard application that harnesses the advanced statistical analysis functions of the R/BioConductor project. Robin implements streamlined workflows that guide the user through all steps of two-color, single-color or Affymetrix microarray analysis. It provides functions for thorough quality assessment of the data and automatically generates warnings to notify the user of potential outliers, low quality chips or low statistical power. The results are generated in a standard format that allows ready use with both specialized analysis tools like MapMan and PageMan and generic spreadsheet applications. To further improve user-friendliness, Robin includes both integrated help and comprehensive external documentation. To demonstrate the statistical power and ease of use of the workflows in Robin, we present a case study, in which we apply Robin to analyze a two color microarray experiment comparing gene expression in tomato leaves, flowers and roots.

INTRODUCTION

Since the first microarray experiments were performed in the 1990's (Schena et al., 1995) a lot of effort has been put into the development of this technique as well as into approaches for the correct analysis of the resulting data. Widespread use of the various array technologies has been accompanied by the development of many sophisticated statistical methods to process the raw data, and to analyze the results in order to infer new biological insights (Sreenivasulu et al., 2006; Usadel et al., 2008; Winfield et al., 2009; Zanor et al., 2009 and see below). The wealth of data and methods leaves the individual researcher with the problem of choosing the correct strategy since it is not directly obvious to the inexperienced user which approach is suitable for a given experimental design. Furthermore, the wide application and technical improvement of microarrays has also resulted in the establishment of large publicly accessible expression data repositories such as GEO, AtGenExpress or GeneVestigator, (Schmid et al., 2005; Barrett et al., 2007). Data mining of these and other public collections is facilitated by descriptive meta data that is attached to the expression data (MIAME and MIAME/Plant (Brazma et al., 2001; Zimmermann et al., 2006), XEML, (Hannemann et al., 2009)). However, choosing the correct approach to statistically (re-)analyze such data also inevitably requires expertise in statistics.

One of the most advanced tools for the analysis of high-throughput experimental data is the statistics environment R. This open source project is constantly being developed and refined by leading statisticians (R Development Core Team, 2008). Together with the R packages provided by the BioConductor project (Gentleman et al., 2004), R provides a powerful, yet flexible, platform for microarray data analysis and quality assessment. The big disadvantage of R/BioConductor-based data analysis however, is its general lack of an intuitive graphical user interface (GUI). The largest part of the functionality of R can only be accessed via a text console. This represents a considerable obstacle for many biologists, who are inexperienced in the use of such interfaces. Furthermore, full

use of the power of R/BioConductor-based data analysis requires programming skills.

Although several GUI applications have been developed that allow analysis of microarray data generated by different technical platforms, these are often commercial (GeneSpring, GeneMaths XT etc), not very intuitive (limmaGUI, affylmGUI, Wettenhall and Smyth, 2004; Wettenhall et al., 2006), not available on all computing platforms (PreP+07, Martin-Requena et al., 2009) or are web-based solutions that would either require uploading of potentially sensitive, unpublished data or laborious local installation such as CARMAWEB or EMMA 2 (Rainer et al., 2006; Dondrup et al., 2009). Although packages like the TM4 suite (Saeed et al., 2003) provide a collection of excellent tools for microarray analysis, it does not offer a consistent, workflow-oriented interface to the user due to its multi-program structure. Additionally, it does not provide support for single color chip platforms like Affymetrix GeneChips without further adaptation.

To address the need for a free, user-friendly and instructive open source tool for microarray analysis, we have developed Robin. Robin provides a Java-based GUI to up-to-date R/BioConductor functions for the analysis of both two-color and single channel (Affymetrix GeneChip) microarrays and implements wizard-like workflows that guide the user through all steps of the analysis including quality assessment, evaluation and experiment design. Robin assists the user in the interpretation of the results by automatically issuing warnings if quality check parameters exceed or undercut conservatively chosen threshold values, or statistical analysis indicates problems like insufficient input data. During the whole workflow the major attention is placed on simplicity and intuitiveness of the graphical user interface. Advanced options to modify the parameters of the analysis functions are, by default, hidden from the user. Naturally, more experienced users have the possibility to activate an expert mode, which allows them to adjust the settings to meet their individual needs, and even review and modify the R scripts before they are executed by the embedded R engine. The

generated output includes informative plots visualizing the quality check and statistical results, the R scripts that have been automatically generated from the users' input, and a complete statistical analysis of the response of gene expression in a form that can directly be imported into common spreadsheet applications, and meta-analysis tools like MapMan for visualization. A detailed user's manual including step-by-step walkthroughs for the different analysis workflows implemented in Robin, examples for all types of quality checks and comprehensive explanations of the statistical settings is available online (http://mapman.gabipd.org/web/guest/tutorials-manuals-etc).

RESULTS AND DISCUSSION

Robin implements standardized workflows for the analysis of common microarray experiment designs, including common reference and direct design two-color experiments and simple multifactorial designs in which more than one experimental condition is being varied.

Installation and scope

Robin is available as standalone installer package including an embedded minimal R engine (plus the required packages) for Microsoft Windows (XP or higher) and Mac OS X (version 10.5 or higher) from http://mapman.gabipd.org/web/guest/robin-download. Installing these packages will leave an existing installation of R on the target system untouched. For all other systems that support Java and R, such as Linux, a lightweight package that can incorporate and configure an existing R installation for usage with Robin is available.

Importing raw data

The user can choose between three separate workflows, specialized for Affymetrix GeneChip, for generic single channel (e.g. Agilent etc) and for two-color microarray data normalization and analysis. Importing Affymetrix GeneChip data is very simple and just requires the user to pick the raw data files that will be

included in the analysis. Since the Affymetrix CEL data format is uniform and does not require further processing or configuration, the user can directly proceed to the quality assessment step. Due to the various file formats in use for non-Affymetrix microarray data, special care has been taken to provide a versatile import wizard that assists the user in the import of arbitrary tabular single- and two-color data. The only restriction imposed is that the data has to be in tabular text format.

The user chooses the chip grid layout from a list of predefined layouts, or enters a custom layout. For convenience, the layouts of several common plant microarrays such as TOM1, TOM2, Medicago16K and Pisum6k (Alba et al., 2004; Hohnjec et al., 2005; Thompson et al., 2005; CGEP; Cornell University, Ithaca, NY, USA) are bundled with Robin as layout presets. All settings of the import wizard interface can be saved as an input data preset to speed up loading of similar data. During the import, Robin tries to automatically separate header information from the tabular data section in the input file and asks the user to specify which columns contain the fields required for analysis (i.e. red channel foreground and background, green channel foreground and background intensities and a unique identifier for each measured signal). When importing single- and two-color data, Robin tries to determine whether the chip layout comprises probes spotted in duplicates. After importing the data, the user is asked to define the 'targets table' by entering the different RNA samples and specifying which sample has been labeled with which dye on each chip. For subsequent analysis, a reference sample must be specified. In very simple experiments that only comprise replicate chips of two different treatments (possibly including dye swaps), Robin uses the first entered sample as reference by default. If data conforming to a common reference design was entered, Robin automatically detects the common reference sample and prompts the user in case this sample was not set as reference. During this step, Robin also analyses the input and tries to make sure that the data is consistent e.g. by verifying that the samples are not disconnected. Import of Affymetrix single channel data does

not cause such problems, since the data format is uniform and it is not necessary to define a targets table.

Quality assessment

After importing the chip data, a variety of quality assessment methods (Fig. 1) can be run, to allow the user to get an overview of the quality of input data and subsequently exclude chips that show strong technical artifacts individually. The various quality assessment methods can be freely chosen and combined as required. For ease of use, robust standards are preselected for the normalization, p-value correction and statistical analysis that yield reliable results in most cases. However, the expert user can choose which normalization, p-value correction and statistical analysis approach (linear model-or rank product-based) to use. These more advanced settings are not displayed by default, but advanced users can take control of analysis parameters and modify them according to their needs.

To support the user in the evaluation of quality assessment results, warnings are issued automatically if quality measures of individual chips exceed conservatively chosen threshold values (see Materials and Methods section for details). Specifically, methods available for quality assessment of single channel data are (I) RNA degradation analysis, (II) box plots and (III) density plots of raw probe signal intensities, (IV) pseudo-images of probe level model (PLM) residuals, (V) scatter plots of the average probe intensity (A) against the logarithmic fold change in expression (M; MA plots), (VI) scatter plots comparing all possible combinations of two individual chips, (VII) visualization of principal component analysis and hierarchical clustering of the normalized expression values (VIII) box plots showing the normalized unscaled standard errors (NUSE) and relative logarithmic expression (RLE) of the probe level models and (IX) false color images of the background signal intensity for non-Affymetrix arrays (see supp. Fig. S1).

PLM-based methods are available for Affymetrix arrays only, while the other functions can also be run on generic single channel chips. Methods available for two–color chip quality assessment are (I) image plots visualizing the chip background signal intensities, (II) density plots of the probe intensity distribution before and after normalization, (III) MA plots of raw and normalized data for each chip and (IV) image plots showing the M value for each probe color coded on a pseudo chip (see supp. Figs. S1 and S6).

All of the above mentioned quality checks have been implemented in R using functions provided by the Bioconductor packages affy, affyPLM, affycoretools, simpleaffy, gcrma, plier, limma, marray and RankProd (Wang et al., 2002; Bolstad, 2004; Gautier et al., 2004; Smyth, 2004; Wu et al., 2004; Affymetrix, 2005; Wilson and Miller, 2005; Hong et al., 2006 and MacDonald, unpublished). Some functions were modified to enhance the visual output. Depending on the type of input data the user can choose between different analysis approaches: In case of single channel data, linear model based (limma) or rank product based (RankProd) analysis is available. Two color data will always be analyzed using limma functions. Quality analysis (QA) results will by summarized in a scrollable list showing clickable thumbnail images of the QA plots. Individual chips showing warnings may be manually excluded from the analysis to prevent them from introducing technical bias in the subsequent assessment of differential gene expression.

Experiment design

When working with Affymetrix data, depending on the statistical analysis strategy chosen, the user can define two (when using rank product) to any number (using limma) of groups of replicates, and assign the imported data files accordingly. Unique labels identifying the groups have to be chosen – these labels will be used later on when defining the contrasts of interest. Robin will generate a warning if groups contain less than three replicates, which can lead to a lower reliability of the results if too few data points are available for the analysis of

differential expression. It should be noted that in the present build of Robin, all replicate experiments are treated as true biological replicates. Entering data that is only technically replicated as an independent replicate will lead to an overestimation of significance when analyzing differential gene expression, however given the reliability of modern microarrays using technical replicates is most often no longer necessary.

Subsequently, the replicate groups are depicted as draggable boxes on the graphical designer panel. This allows the user to visually lay out comparisons of interests between the groups. To achieve this, one simply has to draw an arrow by control-click-dragging from one box to a second box, e. g. from ‘wildtype’ to ‘mutant’ as shown in (Fig. 1). Robin interprets this operation as the comparison ‘wildtype minus mutant’. If more than one experimental condition is being varied, the difference of differences can be extracted using so called ‘interaction terms’. These can be defined by creating ‘meta groups’ and drawing arrows between them (see Fig. 1). Specifically, the operation performed on the meta groups shown in figure 1 will be interpreted as the interaction term ‘(wildtype minus wiltype stressed) versus (mutant minus mutant stressed)’ and will extract those genes that respond to stress differently in mutant and wild type.

The expert settings box included on the experiment designer panel again allows advanced users to change all relevant parameters of the statistical analysis, like p-value- and minimal log2-fold change cutoff, correction method for multiple testing, normalization (although it is not recommended to use different normalization methods for quality control and main analysis) and the statistical strategy for multiple testing across contrasts. Additionally, expert users can choose to review the R script that is generated from the inputs before it is sent to the R engine and include custom code or use Robin to quickly and comfortably generate skeletons of analysis scripts that can then be used as starting points for more sophisticated customized analyses.

Analysis and Results

The statistical methods Robin employs to identify differentially expressed genes are based on two different approaches: Linear modeling (limma, (Smyth, 2004)) and rank product-based analysis (RankProd, (Breitling et al., 2004; Hong et al., 2006)). When analyzing Affymetrix data, the user can choose between these two options, with the restriction that rank product-based inference of differential expression is only available when two groups are to be compared. The two methods differ in the approach they take to the detection of differentially expressed genes. While the linear model-based method relies on advanced statistical modeling and bayesian inference, the rank product approach has a closer resemblance to biological reasoning on the data. For further details on the statistical methods, please refer to Smyth, 2004, (Breitling et al., 2004; Hong et al., 2006) and the Robin Users' Guide available online (http://mapman.gabipd.org/web/guest/tutorials-manuals-etc). Since rank product-based analysis is limited to comparing two experimental conditions, the linear model based analysis offers far more options and flexibility with respect to the available settings and design of the experiment (e.g. if two factors, like genotype and treatment, are being varied in an experiment and the user is interested in the interaction effect).

After collecting all necessary information from the user, Robin generates an R script that is subsequently executed by the embedded R engine. The script produces a comprehensive set of output files that are organized in a folder structure. The results include several informative plots summarizing the statistical analysis: MA plots are created for each comparison, in which the genes that are called as significantly differentially expressed are highlighted in red (see supp. Fig. 2). If less than five comparisons are defined, Robin generates Venn diagrams visualizing the number of genes responding differentially and the overlap of response between contrasts (see Fig. 2). Dendrograms showing the hierarchical clustering of the data based on Pearson correlation of expression, and scatter plots of principal components (PCA) provide an overview of the

internal structure of the data. Robin automatically saves several tables containing the complete statistical analysis for all the genes, and for the top 100 differentially expressed genes for each comparison made. Summary tables that are formatted for direct import and visualization in the meta analysis tools MapMan and PageMan (Usadel et al., 2005; Usadel et al., 2006) allow Robin to be easily integrated with downstream analyses. These files list the log2 fold change in expression for each gene in each comparison, plus a flag denoting the results of the statistical testing (0 = not significantly regulated, 1 = significantly up regulated, -1 = significantly down regulated). These flags can be used for convenient filtering in MapMan (see Usadel et al., 2009 for further details). For Affymetrix data, present and absent calls are calculated using the mas5calls implementation provided by the affy BioConductor package (Gautier et al., 2004). All plots generated in the quality analyses, processed input files, the generated R source code and a short text file summarizing the analysis are written to the output folder to completely document the analysis workflow and ensure reproducibility of the results.

Case study – Comparison of tomato tissues

Robin was used to analyse a data set generated by analysing gene expression in tomato flowers, roots and leaves, using TOM2 microarrays in a two color microarray experiment setup (see the materials and methods section for details). Quality assessment showed that there were no obvious or severe technical artifacts visible on the chips when investigating the background intensity images and the signal intensity distributions plots (supp. Fig. 6). Warnings were generated for all MA plots of the individual chips because of a slightly elevated percentage (between 10.141% and 13.43%) of genes that showed a greater than two fold change in expression.

These warnings are based on the assumption that most of the genes will not show differential expression in any given experiment, and are automatically issued if the percentage exceeds 5%. However, when comparing very different

tissue types, as it is the case in the experiment described in this study, larger differences in gene expression may be expected. Nevertheless, having high percentages of differentially expressed genes runs counter to the initial assumption that most of the genes are not responding, and since the normalization procedure is based on this assumption, normalization might fail. Another reason might be an overestimation of expression values due to an elevated signal to noise ratio. As often observed in two color microarray experiments, the raw signal intensities differ in the red and green channel (see supp. Fig. 6). This technical bias can largely be eliminated by using the standard background subtraction and scaling normalization approach in Robin, as shown on supplementary figure 6. Since none of the chips showed strongly outlying behavior in the quality assessment step, all were included in the statistical analysis of differential gene expression.

The three tomato tissues were compared against each other using a direct design with three biological replicates and dye swaps. In total, 418 genes were found to be significantly differentially regulated between leaves and roots, 200 when comparing leaves to flowers and 234 in the comparison of flowers to roots. As indicated on the Venn diagram (Fig. 2), a substantial number of genes showed differential expression levels in more than one comparison.

The results obtained in Robin were then analyzed using MapMan (Usadel et al., 2009) to gain insights into the biological context of relevant differences in gene expression. Using the biological pathway visualization capabilities of MapMan, general differences could be observed when comparing the aboveground organs with roots. The most prominent changes were, as could be expected, for genes related to photosynthesis. The MapMan BINs (1.1 PS.light reaction, 1.2 PS.photorespiration, 1.3 PS.calvin cycle and 19 tetrapyrrole synthesis) were strongly and very consistently up-regulated in leaf and flower tissue (Fig. 3, supp. Table 2 and supp. Fig. 3) compared to roots. The difference between leaves and flowers was much less pronounced, although still significant. This result can

clearly be attributed to the fact that leaves as the primary sites of photosynthesis supply sink organs like roots and flowers with assimilates and hence need to maintain the photosynthetic machinery in a functional state. These results indicate that the major biological differences were readily identified by Robin and MapMan and prompted us to investigate more subtle differences.

In addition to the visual inspection of pathways provided by MapMan, the built-in Wilcoxon rank sum test function was used on all three comparisons to identify significantly changed MapMan BINs (see supp. Table 2). Other general processes that were found to be significantly upregulated in leaves compared to both flowers and roots included starch synthesis and degradation. In-line with the expectations, sucrose breakdown-related genes like sucrose synthase showed increased expression in roots. Sucrose synthase is presumably involved in sucrose breakdown to provide for carbon supply in sink organs (Sun et al., 1992; Zrenner et al., 1995). Surprisingly, invertases, that are required for normal root growth in *Arabidopsis* (Barratt et al., 2009), showed slightly stronger expression in leaves.

YABBY transcription factors have previously been shown to be involved in the regulation of lateral organ development (Street et al., 2008; Stahle et al., 2009). They were found to be significantly upregulated in leaf (SGN-U603003) and flower tissue (SGN-U591723, SGN-U577176, SGN-U603003, see supp. Fig. 3). The expression of YABBY proteins was strongest in flowers supporting their well-described prominent role in flower development (Fourquin et al., 2007; Ishikawa et al., 2009; Orashakova et al., 2009). Investigation of the development-specific expression pattern of *Arabidopsis* YABBY proteins using the Genevestigator tool (Zimmermann et al., 2004) revealed a similar expression pattern for the CRC (crabs claw) protein showing highest expression in mature flowers (supp. Fig. 4). Similarly, the MADS-box transcription factors showing high similarity to SEPALLATA (SEP1/2) and AGAMOUS-like (AGL8/12) from *Arabidopsis*, that are known to regulate flower and seed development (Mizukami et al., 1996; Pelaz et

al., 2000) also see Robles and Pelaz, 2005 for a review), show strongest expression in flower tissues (see supp. Fig. 3), confirming the fidelity of the results generated using Robin.

MapMan BINs that were primarily upregulated in root tissue included lignin biosynthesis (16.2.1), plasma membrane intrinsic proteins like aquaporins (34.19), and genes related to flavonoid synthesis and metabolism of phenolic compounds. Although the latter two were not significantly responding according to the Wilcoxon rank sum, individual genes showed significant responses. Since expression of flavonoid biosynthesis genes in root tissue is induced in the light (Hemm et al., 2004) the upregulation of SGN-U565166, SGN-U565164 (similar to flanonol synthase) and SGN-U563058 (similar to flavonone-3-hydroxylase) might indicate an artifact due to exposure of the root to light during sample harvesting.

Flower tissue displayed a strong expression of cell wall degrading enzymes like pectin esterase, pectate lyases and polygalacturonases in comparison to both leaves and roots. Pectin methyl esterases (PME) catalyze the demethylation of pectin changing the gelating properties of pectin and making it amenable to cleavage by pectate lyases and polygalacturonases. Apart from their role in simple pectin degradation, recent studies have also shown a prominent role of PMEs in controlling cell adhesion, organ development, and phylotactic patterning (see Wolf et al., 2009 for a recent review). Previous screens of cDNA libraries derived from maize pollen have shown high expression levels of pectin degradation related genes in flower tissues (Wakeley et al., 1998) that are believed to play a role in pollen tube elongation. Interestingly, two putative PMEs (SGN-U585819 and SGN-U585823) exhibited deviating behavior with low expression in flowers. Further investigations using the tomato genome browser provided by the sol genomics network (http://solgenomics.net/gbrowse/gbrowse/ITAG_devel_genomic/) revealed that both genes are located on the same chromosome in direct vicinity of each other possibly indicating that they originate from a tandem duplication event. The

observations reported above were highly significant both on the pathway level, as tested by the wilcoxon rank sum test, and on the level of individual genes as confirmed by the statistical analysis of differential gene expression (please see supp. Table 1 for full details).

MATERIAL AND METHODS

Implementation of Robin

Robin was implemented in Java and R using free extension libraries developed by several software projects. Specifically, the NetBeans visual API (http://graph.netbeans.org/) was used to develop the visual experiment designer, and the AffxFusion (http://www.affymetrix.com/partners_programs/programs/developer/index.affx) library was employed for the extraction of detailed information from Affymetrix chips. Apache commons (http://commons.apache.org/) was used to facilitate generic string operations. To achieve an improved user experience and better integration into the Mac OS X platform, we used the AppleJavaExtensions provided by Apple, Inc., and the QuaQua (http://www.randelshofer.ch/quaqua/) look and feel.

A stand-alone “slim-line” R engine is embedded in the Robin package, and is independent of user installed versions of R. All required BioConductor packages have been included to provide an all-in-one package that works directly after installation. Installer packages for different operating systems were created using the free IzPack installer generator (http://izpack.org/). We also provide a lightweight package without R that can be deployed on any Java-enabled platform. On first use, this version of Robin will ask the user for a path to a working R installation, check this installation and automatically download all required packages (if not already present), provided the computer has a working internet connection.

Automatic input assessment and generation of warnings

Robin tries to aid the user in assessing the quality of the microarray data by automatically generating warnings if diagnostic measures are exceeding preset threshold values. The assessment of global RNA degradation effects as implemented by the AffyRNAdeg function (Gautier et al., 2004) yields slopes for each of the degradation curves. If the slopes of individual RNA degradation curves exceed a value of three or deviate by more than 10% from the median slope of all curves, a warning message indicating the affected chips is displayed in the quality check result list. MA plots visualizing the log2 fold change in expression of gene G under condition C vs. condition D ($M = \log G_C - \log G_D$) plotted against the average log2 probe or probeset intensity ($A = ½ * (\log G_C + \log G_D)$) are generated for each individual chip. In the case of two color microarrays the red channel signal intensity is compared against the green channel signal intensity. To display MA plots for Affymetrix arrays, the normalized expression values of each chip are compared against a synthetic chip created using the median expression values of all probesets across all chips in the experiment. Based on the assumption that most genes will not respond differentially to a given treatment, Robin automatically warns the user if more than 5% of the probesets on an individual chip are more than two fold up- or down regulated. This threshold might be too restrictive in certain experiments e.g. where very different developmental stages of an organism are compared or a drastic treatment is applied. Nevertheless, on data sets that violate the assumption that most genes are not responding, the normalization might fail and introduce artificial effects distorting the original data. Generally, though, a high percentage of differentially responding probesets might indicate artifacts caused e.g. by a low signal-to-noise ratio or large differences in probe signal intensity that could not be eliminated by normalization or even pathogen attack. Again based on the aforementioned assumption, the M values plotted on a MA plots should be centered around M=0. A lowess fit (Cleveland, 1979) is calculated for the MA plots. In the ideal case the lowess fit curve would be identical to the M=0 line. As an estimate for a strong deviation of the lowess fit from the M=0 line, the

area between the lowess curve and the M=0 line is calculated. If the area exceeds a value of 1, a warning will be issued to notify the user of possible artifacts that might be caused by e.g. a bimodal probe signal intensity distribution. Probe signal intensity oversaturation is estimated by calculating the percentage of probes whose raw signal intensity is equal to the highest intensity value measured within that chip. Usually only one or a few probes display maximal intensity (in the case of Affymetrix GeneChips the theoretically possible maximal dynamic range of probe signal intensity is 0 to 2^{16} due to the 16 bit data precision of Affymetrix GeneChip scanning devices). If more than 0.25 % of the probes have maximal intensity, the chip is considered oversaturated and a warning is generated, informing the user of the possible information loss.

Detection of spot replication relies on the spot identifiers and is based on the assumption that if the gene spots are not duplicated but the controls are duplicated, the number of unique identifiers will be greater than 50% of the total number of spots. This should be true for all array types that have more gene spots than control spots, but might not be the case for “boutique” arrays that only contain few probes (e.g. custom arrays designed for small organellar genomes). If replicate spots are detected, Robin sorts the input data by identifier to make sure that replicates are consecutive, sets the number of duplicates to two and the spacing between duplicates to one. Obviously, this is incorrect in cases where more than two replicates are spotted on the array. When analyzing arrays on which the spacing of replicate spots is not uniform, this approach might lead to overestimation of significance and underestimation of correlation for replicate spots that are close together on the array. To account for this possible bias, Robin generates a warning when replicates are detected and informs the user of the assumptions made.

Since the rank product-based analysis does not accept duplicated spots on one array, Robin checks the input data and collapses replicated values identified by the same identifier to the median value within each array. If replication is detected a file containing the replicated spot identifiers and values will be written to disk. In addition to the warnings issued during the quality assessment, Robin will also

inform the user of problems that occurred during the statistical analysis of differential expression, like low or imbalanced numbers of biological replicates and low significance of the results (e.g. none of the probes tested is called significantly differentially expressed given the chosen thresholds). At the end of the analysis workflow, Robin will present a summary list of all generated warnings to ensure that the user is made aware of possible shortcomings of the data.

Plant material

Solanum lycopersicum plants cultivar M82 seeds were allowed to germinate directly on soil and were then transferred to a vermiculte-based groth substrate and further cultivated as described in (van der Merwe et al., 2009). Plant materials for microarray analysis were harvested from 6 week-old plants. Specifically, leaf samples were taken from the third to fourth node from the top, roots were washed in tap water to remove growth substrate and all fully expanded flowers were collected. In order to minimize circadian effects, samples were taken on two consecutive days at the same time of day within 1 ½ hours. Tissue samples were immediately shock frozen in liquid nitrogen and stored at -80°C.

Sample preparation

Tomato RNA extraction was performed using a modification of the standard TRIzol (Invitrogen GmbH, Karlsruhe) extraction protocol. Briefly, 500 mg of frozen material was finely ground in a mortar and subsequently mixed with 5 ml of TRIzol solution by vortexing. After addition of 3-5 ml chloroform and centrifugation for 20 minutes at 4000xg, the aqueous phase containing the RNA was transferred to a fresh tube. RNA was precipitated over night following addition 0.5 volumes of precipitation solution (0.8 M sodium citrate, 1.2 M sodium chloride) and 0.5 volumes of 2-propanol. Precipitated RNA was recovered by centrifugation for 20 minutes at 4000xg and subsequently washed twice by adding 5 ml of 70% ethanol and centrifuging for 5 minutes at 4000xg. After complete removal of 70% ethanol, the RNA pellets were air-dried and finally

dissolved in 40 µl of sterile water. cDNA synthesis and labeling was carried out as described in (Degenkolbe et al., 2005) using Dynabeads Oligo(dT)25 (Dynal, Oslo, Norway) to extract mRNA from the whole RNA samples.

Chip hybridization and data processing

The TOM2 microarrays were obtained from the Boyce Thompson Institute (Ithaca, NY, USA). Each microarray contains 11890 oligonucleotide probes designed based on gene transcript sequences from the Lycopersicon Combined Build # 3 unigene database (http://www.sgn.cornell.edu). Chip hybridization was performed as described in (Degenkolbe et al., 2005) with the following modifications: Total RNA was extracted according to Bugos et al. (1995) with minor modifications. The slides were rehydrated over a 65°C waterbath for 10 sec and UV-cross-linked at 65 mJ. The pre-hybridization was performed for 45 min at 43°C in 5x SSC, 0.1%SDS, 1% BSA, washed twice for 10 sec in milliQ water (Millipore) and in isopropanol for 5 sec and drained by centrifugation at 1500 rpm for 1 min. After hybridization the slides were washed in 1x SSC, 0.2% SDS for 3 min at 42°C and 3 min at room temperature; after that the slides were washed again in 0.1x SSC, 0.2% SDS for 3 min at room temperature, three times in 0.1x SSC for 3 min at room temperature. The arrays were then drained by centrifugation at 1500 rpm for 2min. All three possible comparisons between the three tissues were performed in three biological replicates resulting in nine microarray hybridizations. Raw signal intensity values were computed from the scanned array images using the image analysis software GeneSpotter version 2.3 (MicroDiscovery, Berlin, Germany). The raw intensity values were normalized using Robin's default settings for two color microarray analysis. Specifically, background intensities estimated by GeneSpotter were subtracted from the foreground values and subsequently a printtip-wise loess normalization (Yang et al., 2002) was performed within each array. To reduce technical variation between chips, the logarithmized red and green channel intensity ratios on each chip were subsequently scaled across all arrays (Yang et al., 2002; Smyth and Speed, 2003) to have the same median absolute deviation. Statistical analysis of

differential gene expression was carried out using the linear model-based approach developed by (Smyth, 2004). The obtained p-values were corrected for multiple testing using the strategy described by (Benjamini and Hochberg, 1995) separately for each of the comparisons made. Genes that showed an absolute log2-fold change value of at least 1 and a p-value lower than 0.05 were considered significantly differentially expressed. The log2-fold change cutoff value was imposed to account for noise in the experiment and make sure that only genes that show a marked reaction are recorded. The TOM2 chip oligonucleotide annotation was updated based on BLAST (Altschul et al., 1990) searches against the newest version of the SGN tomato unigene set (Tomato 200607 build2, http://solgenomics.net/) and MapMan BINs were assigned to each oligonucleotide on the chip based on the SGN tomato unigene mapping. Wilcoxon rank sum tests were performed to test whether there were bins that were significantly and consistently behaving different than the other bins in the MapMan ontology using the built-in function in MapMan.

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FIGURE LEGENDS

Figure 1: (A) Screenshot of the quality assessment functions available for Affymetrix (R) chips. All methods can be freely combined to obtain an overview of the input data quality. Short inline explanations for each method are displayed in the info field on the left side upon clicking the question marks. The expert panel at the bottom of the user interface is providing more option for customizing the analysis settings. By default, robust analysis methods are predefined and panel is hidden to provide a less cluttered interface to inexperienced users. (B) Screenshot of the graphical experiment designer panel. Comparisons between the previously defined groups of biological replicate chips can be configured by dragging visual connections between them. The arrowhead defines the direction of the comparison. E.g. the arrow between the ‘wildtype’ group and the ‘wildtype stress’ group is interpreted as the ‘wildtype - wildtype stress’ contrast, meaning that genes showing a higher expression level in the ‘wildtype stress’ group will have a negative log2 fold change value in the output and vice versa. Interaction terms can be defined via ‘metagroups’, shown as orange boxes.

Figure 2: Venn diagram showing the numbers of genes called significantly differentially expressed when comparing tomato leaf, flower and root tissue. The numbers include both up- and downregulated genes. Genes that are differentially regulated in more than one comparison are depicted in the overlapping areas. As indicated by the number in the lower right corner, 10531 genes were not significantly affected.

Figure 3: PageMan analysis of the tomato case study. A Wilcoxon test was performed, analogous to the test implemented in MapMan, to identify significantly differentially regulated MapMan bins. Individual bins that show distinct responses are highlighted. The plot shows the color coded Z scores of the p-values computed in the test.

SUPPLEMENTAL MATERIAL

Supplementary Material S1: Complete analysis results of the case study as described in the text, including the processed raw microarray data.

Supplementary Material S2: Robin Users' Guide.

Supplementary Material S3: Raw microarray data files of the case study experiment.

Supplementary figure S1: Exemplary overview of the quality assessment plots generated by Robin. All plots have been generated using publicly available data sets obtained from the Gene Expression Omnibus online repository. Specifically, an Affymetrix ATH1 dataset that was published by Morinaga *et al.*, 2008 (GEO accession no. GSE9799) and a TOM1 dataset published by Kolotilin *et al.*, 2007 (GEO accession no. GSE6041) were used. The Affymetrix dataset contains one chip that has been hybridized to genomic DNA and hence shows clearly outlying behaviour in most of the quality checks. (A) Box plot of the probe signal intensities in each chip. The genomic DNA sample GSM246369 shows a deviating distribution indicating a possible technical problem. (B) Box plot of the relative logarithmic expression values. Again, sample GSM246369 is clearly visible as an outlier having a stronger spread. (C) Box plot of the normalized unscaled standard errors of the probe level models (NUSE). (D-F) False color images of the weights applied to each probe on three individual chips. Strong green color indicates stronger down-weighting due to a probe behaviour that strongly deviates from the model. (D) Shows a high quality chip that has consistently high weights, (E) shows a chip with spatially confined regions that have been down-weighted, possible due to washing artifacts, (F) displays strongly deviating behaviour on all probes on the chip and hence was globally down weighted. (G-H) MA plots visualizing the average log2 intensity A plotted against the log2-fold change in expression M of samples GSM246371 (G) and

GSM246369 (H) plotted against the average A and M of all chips in the experiment. The values on plot (G) show an expected distribution with most M values close to zero (i.e. most of the transcripts do not respond differentially) while plot (H) show strong aberrations. (I) Plot of the signal intensity distribution of all chips. Analogous to (A), this plot shows that the probe signal distribution of the genomic DNA sample deviates from the RNA samples and is markedly shifted towards lower values. (J) RNA degradation assessment: Plot of the probe-wise signal ordered from 5'-most probe to 3'-most probe. Usually RNA degradation is more rapid at the 5' end of the molecules. Hence the expected result is an almost linear curve showing higher values at the 3' end. The slope of this curve reflects the degree of degradation. Generally, all RNA degradation curves should be in agreement. Sample GSM246369 shows strong deviations from the other curves due to the different nature of DNA degradation. (K-L) Pseudo images of the red and green channel background signal intensity of sample GSM140124 (K) and sample GSM140127 (L) taken from the TOM1 dataset. On a high quality chip, the background signal intensity should be low and smooth in both color channels as it is the case on (K). Panel (L) shows two different possible problems: 1) A clear blotch of higher background signal in the red channel (indicated by the arrow) and 2) a globally strongly increased green background intensity. While the global increase of the green channel background can usually be eliminated by the normalization, the spatially confined red blotch might impair the accuracy of the measurement of the affected spots. Examples for single color background signal image plots, principal components analysis and hierarchical clustering were not included in the examples shown. Please also see the comprehensive Robin User's Guide for examples of all quality check plots and additional in-depth documentation (http://mapman.gabipd.org/web/guest/tutorials-manuals-etc).

Supplementary figure S2: MA plots of the three comparisons made in the tomato case study experiment. The plots show the average signal intensity (A) and the

average normalized log2-fold change (M) individually for each comparison. Genes showing significant differential regulation are highlighted by red circles.

Supplementary figure S3: Exemplary visualization of the most strongly reacting bins using MapMan. Genes that are not significantly regulated are greyed out using the built-in filter function. The comparisons shown are (A) Leaf – Root, (B) Flower – Root and (C) Leaf – Flower.

Supplementary figure S4: Expression patterns of three YABBY transcription factor homologs from *Arabidopsis* created using the Genevestigator web application. The Affymetrix probe set identifiers correspond to the following YABBY genes: 245029_at: YABBY family protein At2g26580; 260355_at:Crabs claw (CRC) protein At1g69180; 262989_at: Inner no outer (INO) protein At1g23420.

Supplementary figure S5: Genomic locations of two putative pectin methyl esterases from tomato (SGN-U585819 and SGN-U585823) as shown by the Gbrowse genome browser (http://solgenomics.net/gbrowse/gbrowse/ITAG_devel_genomic/). The genes are located on the same chromosome within a range of less than 10kb possibly indicating that they originate from a genetic duplication event.

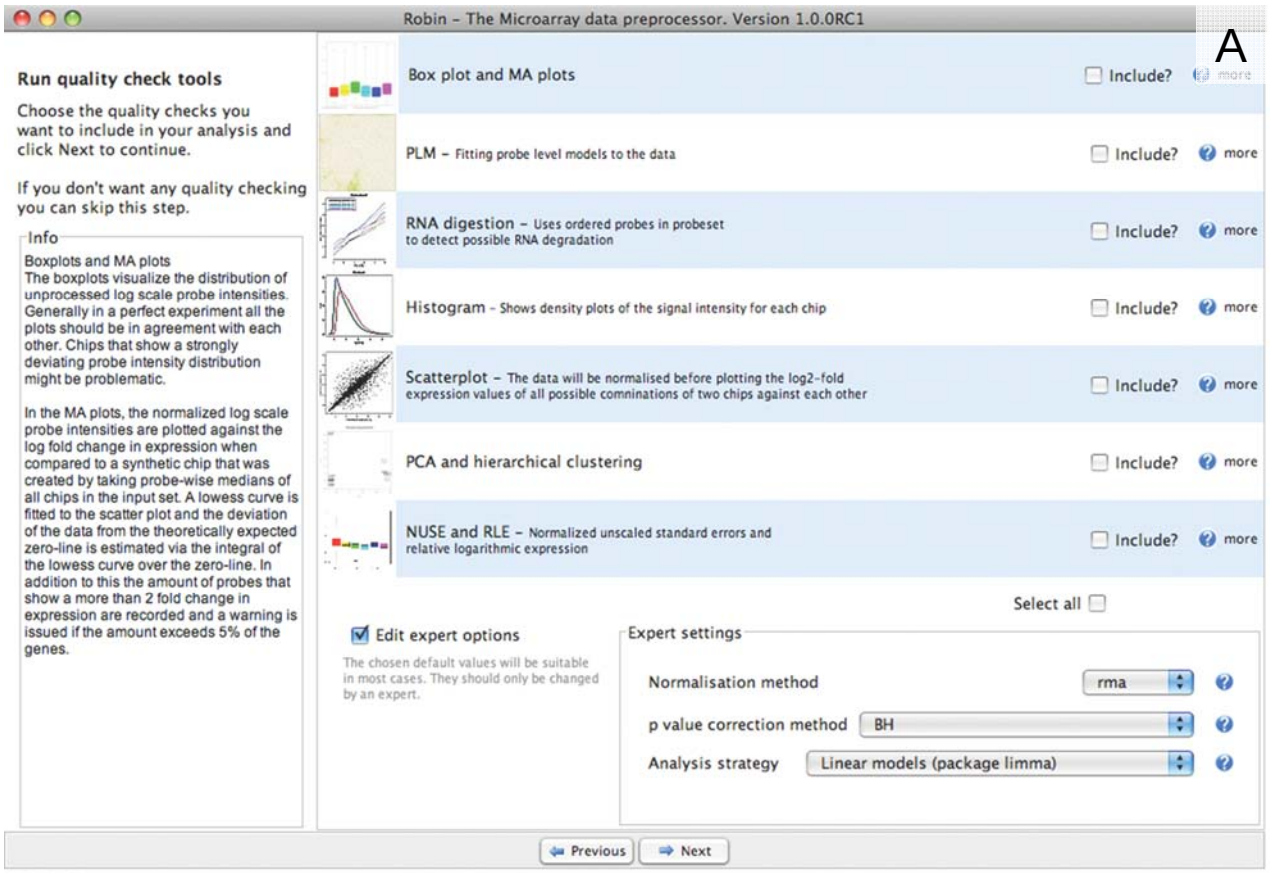
Supplementary figure S6: Summary of all quality check plots generated for the tomato case study experiment. (A) Image plots of the background signals measured on each chip. (B) Chipwise MA plots; (C) False-color images of the log2 ratios of raw red and green channel signal intensities; (D) Overview plots showing the raw and normalized signal intensity distributions on all chips. The upper panel shows density plots and the lower panel shows boxplots of the same values.

Supplementary table S1: Detailed statistical results tables as produced by Robin.

For convenience, the individual tables have been combined into one MS Excel file containing the original tables on separate worksheets. A second set of work sheets has been included that also contains the MapMan bins associated with each of the oligonucleotides on the TOM2 chip and the annotation of the target transcripts taken from the latest tomato unigene release (Tomato 200607 build2). The columns contain from left to right: (Feature.ID) A unique identifier for the oligonucleotide probes or probe sets on the chips; (logFC) the log2-fold change in expression; (AveExpr) average normalized expression value; (t) t-statistic; (P.Value, adj.P.Val) raw and Benjamini-Hochberg-corrected p-values for differential expression; (B) the log-odds for differential expression.

Supplementary table S2: Wilcoxon rank sum test results generated by MapMan. The ‘Elements’ column refer to the total number of genes classified into the respective MapMan bin. P-values denote the probability that the corresponding bin was incorrectly classified as significantly regulated.

Figure 1


Robin – The Microarray data preprocessor. Version 1.0.0RC1
A
Run quality check tools
Choose the quality checks you want to include in your analysis and click Next to continue.
If you don't want any quality checking you can skip this step.
Info
Boxplots and MA plots
The boxplots visualize the distribution of unprocessed log scale probe intensities. Generally in a perfect experiment all the plots should be in agreement with each other. Chips that show a strongly deviating probe intensity distribution might be problematic.
In the MA plots, the normalized log scale probe intensities are plotted against the log fold change in expression when compared to a synthetic chip that was created by taking probe-wise medians of all chips in the input set. A lowess curve is fitted to the scatter plot and the deviation of the data from the theoretically expected zero-line is estimated via the integral of the lowess curve over the zero-line. In addition to this the amount of probes that show a more than 2 fold change in expression are recorded and a warning is issued if the amount exceeds 5% of the genes.
Box plot and MA plots
Include?
PLM – Fitting probe level models to the data
RNA digestion – Uses ordered probes in probeset to detect possible RNA degradation
Histogram – Shows density plots of the signal intensity for each chip
Scatterplot – The data will be normalised before plotting the log2-fold expression values of all possible comninations of two chips against each other
PCA and hierarchical clustering
NUSE and RLE – Normalized unscaled standard errors and relative logarithmic expression
more
Select all
Edit expert options
The chosen default values will be suitable in most cases. They should only be changed by an expert.
Expert settings
Normalisation method
rma
p value correction method
BH
Analysis strategy
Linear models (package limma)
Previous
Next

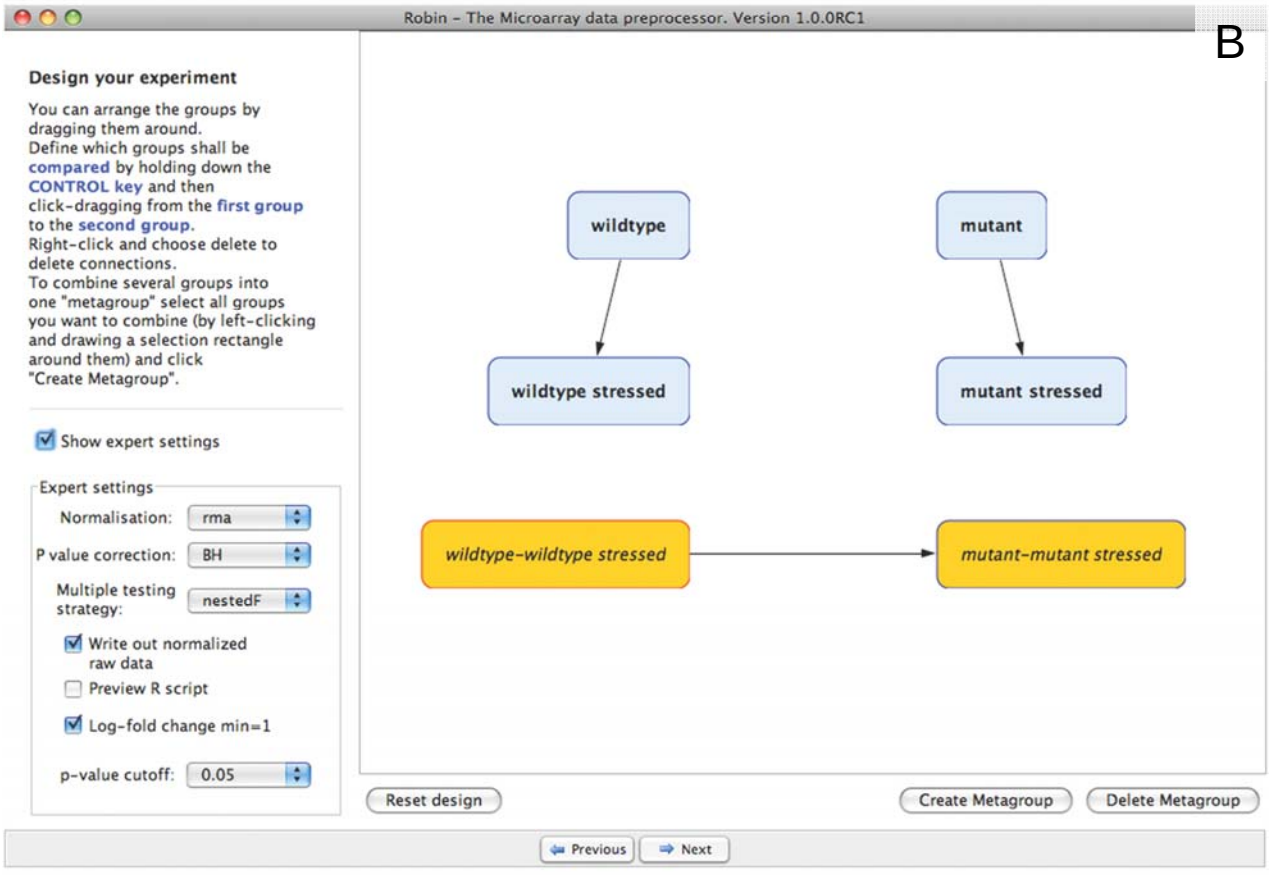

Robin – The Microarray data preprocessor. Version 1.0.0RC1
B
Design your experiment
You can arrange the groups by dragging them around.
Define which groups shall be compared by holding down the CONTROL key and then click-dragging from the first group to the second group.
Right-click and choose delete to delete connections.
To combine several groups into one "metagroup" select all groups you want to combine (by left-clicking and drawing a selection rectangle around them) and click "Create Metagroup".
Show expert settings
Expert settings
Normalisation:
rma
P value correction:
BH
Multiple testing strategy:
nestedF
Write out normalized raw data
Preview R script
Log-fold change min=1
p-value cutoff:
0.05
wildtype
mutant
wildtype stressed
mutant stressed
wildtype-wildtype stressed
mutant-mutant stressed
Reset design
Create Metagroup
Delete Metagroup
Previous
Next

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Figure 2

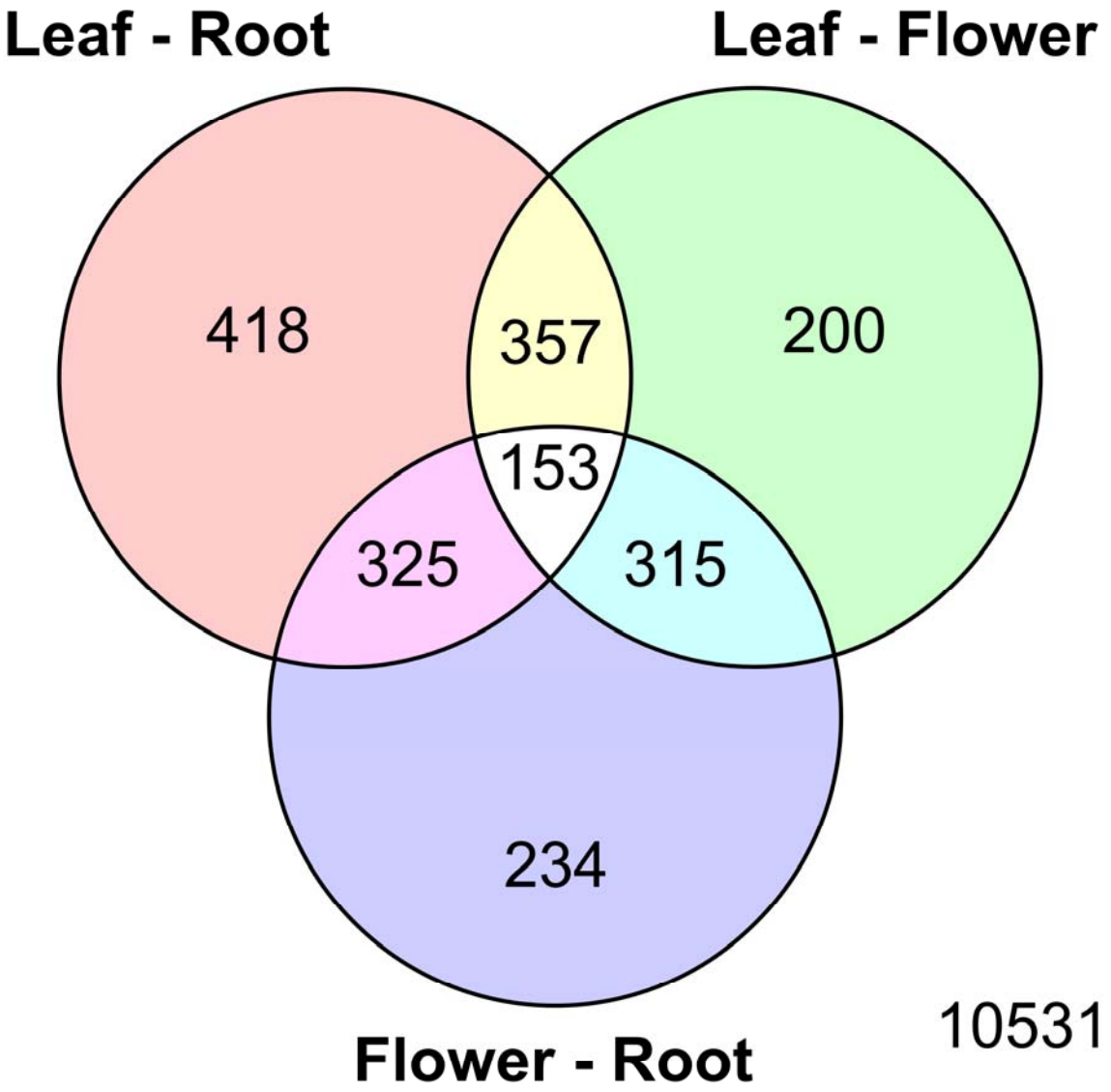

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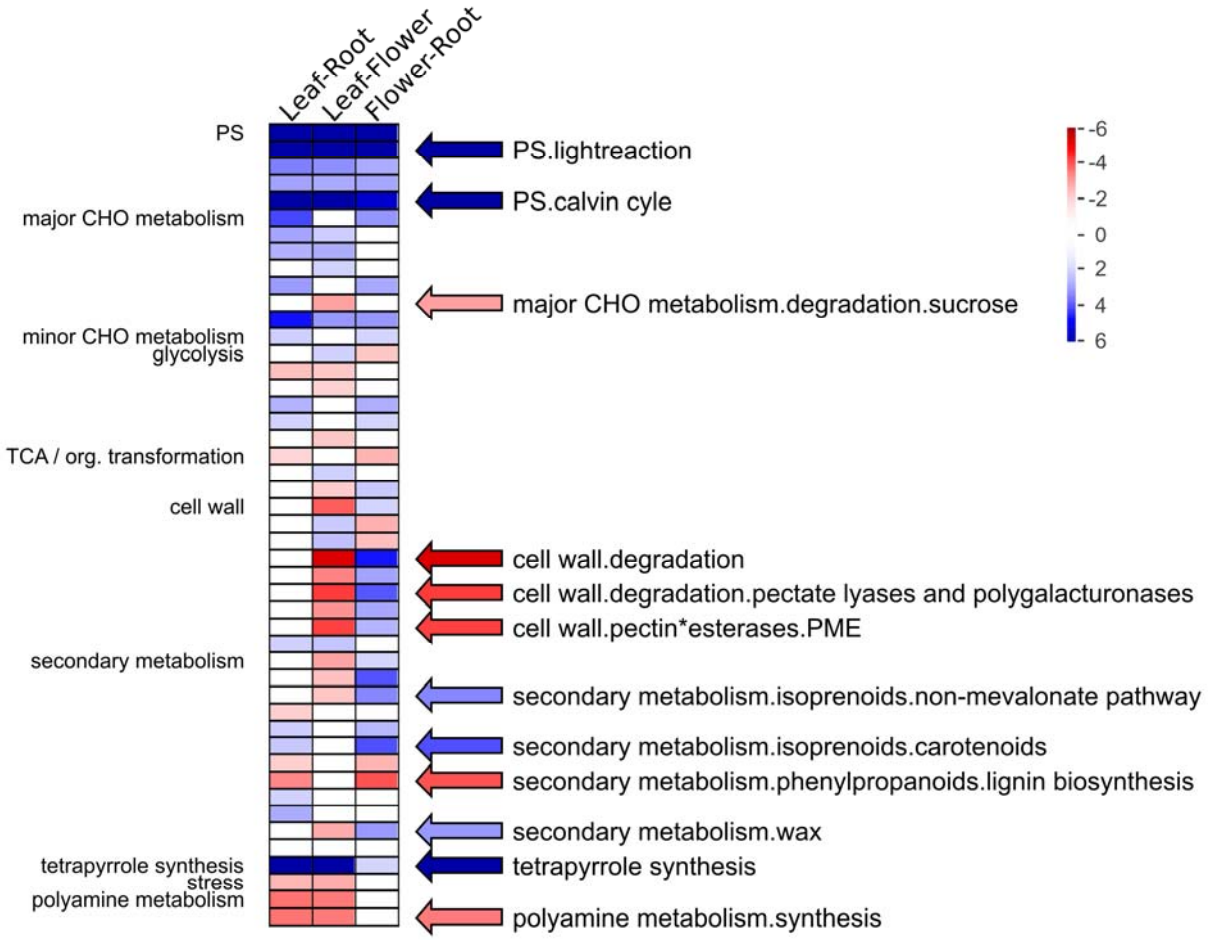
Figure 3



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