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**Molecular signatures of increased freezing tolerance due to low temperature memory in Arabidopsis**

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**Running title:** Cold memory in Arabidopsis

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## ABSTRACT

Alternating temperatures require fast and coordinated adaptation responses of plants. Cold acclimation has been extensively investigated and results in increased freezing tolerance in *Arabidopsis thaliana*. Here we show that the two *Arabidopsis* accessions Col-0 and N14 that differ in their freezing tolerance, showed memory of cold acclimation, i.e. cold priming.

Freezing tolerance was higher in plants exposed to cold priming at 4°C, a lag phase at 20°C and a second triggering cold stress (4°C) than in plants that were only cold primed. To our knowledge this is the first report on cold memory improving plant freezing tolerance. The triggering response was distinguishable from the priming response at the levels of gene expression (RNA-Seq), lipid (UPLC/MS) and metabolite composition (GC-MS).

Transcriptomic responses pointed to induced lipid, secondary and stress metabolism in Col-0 and growth-related functions in N14. Specific accumulation of lipids included arabidopsides with possible functions as signaling molecules or precursors of jasmonic acid. While cold induced metabolites such as raffinose and its precursors were maintained in N14 during the lag phase, they were strongly accumulated in Col-0 after the cold trigger. This indicates genetic differences in the metabolic regulation of cold memory.

**Keywords:** *Arabidopsis thaliana*, Cold acclimation, Cold memory, Freezing tolerance, Lipidomics, Metabolomics, Stress priming, Transcriptomics

## INTRODUCTION

Plants, as sessile organisms, have to respond continuously to changing environmental conditions including recurring stress events. Plants from temperate and boreal climates experience large seasonal temperature variation. Due to global climatic changes winter periods become shorter and early warm temperatures in spring are often followed by sudden cold spells.

Cold acclimation is a plant response to low, non-freezing temperatures that results in improved freezing tolerance. In addition, a tightly regulated deacclimation process, resulting in reduced freezing tolerance in spring, is necessary for the transition to growth and flowering (Vitasse, Lenz & Korner, 2014, Xin & Browse, 2000). At the same time maintenance of freezing tolerance may be important for plants to be prepared for recurring cold periods. On balance, if the transition to reproductive growth is made too late, plants lose valuable time during the growth season, while a premature transition involves the danger of freezing damage during a late-season cold spell (Augspurger, 2013, Marino, Kaiser, Gu & Ricciuto, 2011). Ideally, plants should be rapidly able to grow and develop under warm conditions, but at the same time they should still be prepared for a late frost event.

In this context, priming is an important mechanism of plant adaptation to complex and rapidly changing environments because it provides preparation for a future stress event (Conrath, 2006) and avoids investment of resources into prolonged maintenance of the acclimated state (Hilker *et al.*, 2016). When plants experience a non-lethal abiotic or biotic stress they might get primed for a response to a second triggering stress event after a stress-free lag or memory phase. Priming can result in a level of stress resistance that is superior to the non-primed state (Galis, Gaquerel, Pandey & Baldwin, 2009, Hilker *et al.*, 2016). During the lag or memory phase information about the first stress event may be stored for a limited time to minimize associated costs. However, grasses can retain a drought stress memory that

may last over an entire vegetation period to facilitate better protection during recurrent drought events through improved photoprotection (Walter *et al.*, 2011).

An important feature of stress memory is better protection from a recurring stress without the potentially high metabolic costs associated with constitutive expression of acclimation and stress response genes (van Hulst, Pelsers, van Loon, Pieterse & Ton, 2006).

On the other hand, maintenance of memory that lasts too long might still incur a minor cost for the plants, especially if memory, however slightly, inhibits growth and further development. Therefore, under certain conditions re-establishment of stress tolerance with a rapidly quenched memory of a past stress event could be more favorable.

Although the characterization of determinants of memory formation in organisms without a central nervous system is still in its infancy, some mechanisms have been established mostly from biotic stress triggering (Hilker *et al.*, 2016). These include calcium signaling, changed levels of signaling metabolites or transcription factors, epigenetic effects such as DNA methylation, histone modifications (e.g. histone H3K4 hyper-methylation), regulation of nucleosome occupancy, modification of key regulatory proteins and phosphorylation of mitogen activated kinases, changes in phytohormone levels and alterations of primary metabolism (Bäurle, 2017, Bruce, Matthes, Napier & Pickett, 2007, Conrath, 2011, Conrath, Beckers, Langenbach & Jaskiewicz, 2015, Crisp, Ganguly, Eichten, Borevitz & Pogson, 2016, Galis *et al.*, 2009, Kinoshita & Seki, 2014, Pastor, Luna, Mauch-Mani, Ton & Flors, 2013, Schwachtje, Fischer, Erban & Kopka, 2018).

The process of cold acclimation has been extensively investigated, in particular in *Arabidopsis thaliana* (for reviews see Chinnusamy, Zhu & Zhu, 2007, Gilmour, Fowler & Thomashow, 2004). It involves a multitude of adaptations including induction of CBF transcription factors (C-repeat binding factors) and their down-stream genes, lipid remodeling, accumulation of compatible solutes and COR (Cold Regulated) proteins (Hinch,

Espinoza & Zuther, 2012, Xin & Browse, 2000). On the other hand, the molecular mechanisms underlying the regulation of the loss of freezing tolerance during the lag or memory phase are only poorly understood, despite their ecological and agronomic importance (Zuther, Juszczak, Lee, Baier & Hinch, 2015). Changes in the transcriptome have been reported for a lag phase of 1 to 24 h (Oono *et al.*, 2006) or for three days (Pagter *et al.*, 2017, Zuther *et al.*, 2015) after priming at 4°C, or for three days after priming at 0°C (Byun, Koo, Joo, Ha-Lee & Lee, 2014). A role of the plastid antioxidant system in cold memory to control ROS signaling and oxidative burst has been postulated in Arabidopsis and winter wheat (Li *et al.*, 2014, van Buer, Cvetkovic & Baier, 2016). The increased freezing tolerance upon triggering after a memory phase may also be incomplete as shown for winter wheat that reached only 39% of its primed freezing tolerance after triggering due to a reduced accumulation of carbohydrates, in contrast to canola which showed the same freezing tolerance after priming and after triggering (Trischuk, Schilling, Low, Gray & Gusta, 2014).

Here we selected the differently freezing tolerant Arabidopsis accessions Col-0 and N14 (Zuther, Schulz, Childs & Hinch, 2012) to investigate their ability to remember a cold treatment. Memory of cold exposure lead to enhanced freezing tolerance after a second cold triggering treatment. We investigated molecular the molecular signatures of this memory at transcript (RNA-Seq), lipid (UPLC-MS) and primary metabolite levels (GC-MS). Different response patterns after triggering were observed for the two accessions and for different classes of molecules. Our study contributes novel knowledge on memory and triggering processes in plants and will be the basis for targeted approaches to identify molecular regulators of plant cold memory in relation to freezing tolerance.

## **MATERIALS AND METHODS**

### **Plant material and growth conditions**

The *Arabidopsis thaliana* accessions Columbia-0 (Col-0) and Sampo Mountain (N14) that show intermediate and high freezing tolerance after cold acclimation (Zuther *et al.*, 2012) were used for all experiments. Col-0 originally derives from Poland (52.73° N, 15.15° E) and N14 from Russia (61.1° N, 34.5° E). Plants were initially grown on soil in a climate chamber with 20°C/60% relative humidity (RH) during the day and 6°C/70% RH during the night in a 14 h/10 h light/dark cycle with a light intensity of 180  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . After one week conditions were changed to 20°C/16°C day/night temperature at 60%/75% RH, 8 h day length at 180  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for another week when plants were pricked (10 plants per 10 cm diameter pot). After pricking, the plants were kept under the same conditions for one week and were then transferred to long-day conditions with 16 h at a light intensity of 200  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  for an additional week. These 28 days old plants were cold treated at a constant temperature of 4°C at 16 h day length with a light intensity of 90  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and 70-80% RH for three days (Rohde, Hinch & Heyer, 2004, Zuther *et al.*, 2012). This lower light intensity at the low temperature was used to avoid photoinhibition under these conditions. For a lag or deacclimation phase, plants were transferred for seven days back to the long-day conditions described above (Pagter *et al.*, 2017, Zuther *et al.*, 2015). The cold triggering treatment was applied by transferring the plants back to the cold conditions for three days. To compensate for the one week of development under warm conditions during the lag phase, we also grew plants for an additional week under these conditions before the cold priming treatment (35 days old developmental control).

Samples were taken at the end of the initial growth period from control plants after either 28 days (C28) or 35 days (C35), after three days of cold priming (C28P3, C35P3), after the lag phase (C28P3L7) and after three days of triggering (C28P3L7T3) (Fig. 1). Five replicates containing either 14 to 15 rosettes (C28, C28P3) or four rosettes (all other conditions) were

harvested 7 h into the light phase. Samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The experiment was performed in three independent biological replicates.

### **Determination of plant freezing tolerance**

Freezing damage was determined at all sampling time points (Fig. 1) as electrolyte leakage from conductivity measurements after freezing of detached whole rosettes (C28, C28P3) or half rosettes (all other conditions) to temperatures ranging from  $-1^{\circ}\text{C}$  to  $-16^{\circ}\text{C}$  for plants under control conditions and plants after the seven day lag phase and from  $-1^{\circ}\text{C}$  to  $-20^{\circ}\text{C}$  for primed or triggered plants.  $\text{LT}_{50}$  was calculated from the leakage values as previously described (Thalhammer, Hinch & Zuther, 2014). Five replicates were measured per condition, accession and temperature point. Significant differences among the different treatments within the accessions were evaluated by two-way Analysis of Variance (ANOVA), using the HSD.test from the R package *agricola* for the grouping and TukeyHSD from the package *stats* to calculate p-values.

### **RNA extraction and sequencing**

Homogenized plant material of five replicate samples per accession and treatment was pooled to 100 mg separately for the three independent biological experiments which resulted in 36 samples for the analysis. Total RNA was isolated using a TRIzol protocol based on the 'single step' method (Chomczynski & Sacchi, 2006) with the modifications described recently (Sprenger *et al.*, 2016). Four  $\mu\text{g}$  of each RNA sample was treated with DNase (RapidOut DNA-removal Kit, Thermo Scientific). Absence of genomic DNA contamination was confirmed by qRT-PCR using intron-specific primers (Zuther *et al.*, 2012). Finally, RNA

quality and integrity were verified with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Library preparation and RNA sequencing was performed at the Max Planck-Genome-Centre Cologne, Germany (<https://mpgc.mpiiz.mpg.de/home/>). Libraries were prepared with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) and sequenced using Illumina HiSeq 3000 technology generating 150 base pair (bp) long single end reads. RNA-Seq raw data are available at GEO (<http://www.ncbi.nlm.nih.gov/geo>) under the accession number GSE112225.

### **Bioinformatic processing and differential gene expression (DGE) analysis**

The quality of the pre-processed sequence reads was checked with the FastQC tool, version 0.11.5 (Babraham Institute, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Adapter trimming was done with flexbar, version 2.5 (Dodt, Roehr, Ahmed & Dieterich, 2012) (<http://www.mdpi.com/2079-7737/1/3/895>) and reads smaller than 80 bp were removed. Read mapping was done against the genomic reference of *Arabidopsis thaliana* accession Col-0. The genomic fasta sequence, cDNA and GTF annotation files were downloaded from EnsemblPlants, version TAIR10, release 31 (<http://plants.ensembl.org/info/website/ftp/index.html>). The STAR software, version 2.5.2a (Dobin *et al.*, 2013) was used for read mapping with the following parameters: `--outSAMtype BAM SortedByCoordinate, --outFilterMultimapNmax 20, --alignSJDBoverhangMin 8, --outSAMunmapped Within` and `--quantMode TranscriptomeSAM GeneCounts`.

Prior to the DGE analysis lowly expressed genes with a sum smaller than five counts per gene for all samples were removed, leaving 24,574 genes out of 33,602 with annotations in TAIR10 for further analysis. For the DGE analysis 11 different comparisons were performed

for both accessions (Table 1) with the R-Package *DESeq2* using R version 3.4.2 (RCoreTeam, 2017) and RStudio version 1.1.383 (RStudioTeam, 2015). Normalization for the datasets of the two accessions was performed with the included *DESeq2* approach (Love, Huber & Anders, 2014). For the analysis the batch effect and condition were considered and the parameter `fitType` was set to `local`. Resulting p-values were corrected for multiple testing errors (Benjamini & Hochberg, 1995). The cut-off for DGE was set at  $FDR < 0.1$  and an absolute  $\log_2FC$  of  $> 1$ . Principal Component Analysis (PCA) was performed using the packages *DESeq2* and *ggplot2* after regularized log transformation (`rlog`) of the count data by the `rlog` function of *DESeq2*.

### **Functional enrichment analysis**

Functional enrichment analysis of MapMan annotation bins among significantly differentially expressed genes (<http://mapman.gabipd.org/web/guest/mapmanstore>) was performed with CorTo, version 1.03 (Giorgi, Del Fabbro & Bolger, 2013). P-values were obtained by Fisher's exact test followed by correction for multiple testing (Benjamini & Hochberg, 1995). A p-value threshold of 0.01 was applied and p-values were converted to z-scores using the inverse normal cumulative distribution function. Bin hierarchies higher than three were discarded. The p-values were treated as two-tailed and visualized with the *heatmap.2* function in R.

### **Lipid extraction and UPLC/MS analysis**

Lipid extraction and analysis was performed for five biological replicates per accession and condition and three independent experiments (n=15) as previously described (Salem, Jüppner, Bajdzienko & Giavalisco, 2016). Briefly, lipids were extracted from 20 mg of homogenized tissue by suspending the material in 1 mL of pre-cooled ( $-20^{\circ}\text{C}$ ) MTBE-extraction solution (methanol: methyl *tert*-butyl-ether (1:3; v/v) (Biosolve, Netherlands)),

spiked with 0.5 µg/mL 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine. The samples were incubated for 30 min on an orbital mixer at 4°C followed by sonication for 10 min in an ice-cooled sonication bath. After adding 500 µL of methanol:water (1:3, v/v) to induce phase separation, the samples were vortexed and centrifuged for 5 min at 20,000 x g at 4°C. An aliquot of 500 µL was collected from the upper phase containing the lipids and dried in a vacuum concentrator. The pellet was re-suspended in 250 µL acetonitrile:2-propanol (7:3, vol/vol), of which 2 µL were subjected to UPLC/MS analysis (Salem *et al.*, 2016).

### **Lipid annotation and statistical analysis**

The UPLC/MS data was processed using Progenesis QI for metabolomics (version 2.3; Nonlinear Dynamics, Newcastle upon Tyne, UK). To remove noise and contaminants, data for every lipid species with average peak height lower than the average peak height of the method blanks or with 50% of the values below 1000 arbitrary counts were removed from the data set. Remaining peaks were then assigned to annotated lipid species using an in-house generated lipid database for Arabidopsis (Hummel *et al.*, 2011). The data were normalized to the internal standard and sample fresh weight. For some lipid species, more than one peak was detected having the same m/z and identical adducts but different retention times. In these cases, we added the letter A, B, C or D to the compound name, depending on their elution order. In total, 162 lipids were identified from both accessions. All lipid data are available in Suppl. Table 1.

Normalized mass spectral intensities were log<sub>2</sub> median transformed to approximate normal distribution and represent relative lipid abundance measures. PCA was executed with the R-package *pcaMethods* including Pareto scaling and centering (Stacklies, Redestig, Scholz, Walther & Selbig, 2007). Statistical significance of differences between treatments in lipid pool sizes for 11 different comparisons (Table 1) was tested for both accessions by t-test

at  $P < 0.05$  with correction (Benjamini & Hochberg, 1995) for multiple testing in R. The overlap between lipids with significantly different pool sizes in different comparisons was illustrated by Venn diagrams (R-package *VennDiagram*).

### **GC-MS metabolite profiling**

● Polar metabolites were extracted from 80 mg of ground rosette material from five replicates per experiment and three independent experiments (n=15). Metabolites were analysed by gas chromatography coupled to electron impact ionisation time-of-flight mass spectrometry (GC/EI-TOFMS) using both split and splitless sample injection to enable profiling of both highly abundant and trace metabolites (Dethloff *et al.*, 2014).

### **Metabolite annotation and statistical analysis**

Chromatograms were acquired and baseline corrected by ChromaTOF software (LECO Instrumente GmbH, Mönchengladbach, Germany). Manually supervised identification of metabolites used TagFinder (Luedemann, Strassburg, Erban & Kopka, 2008), the NIST08 software, (<http://chemdata.nist.gov/dokuwiki/doku.php?id=start>) and the mass spectral and retention time index reference collection of the Golm Metabolome Database (Hummel, Strehmel, Selbig, Walther & Kopka, 2010, Kopka *et al.*, 2005). All mass spectral intensities were normalized to fresh weight and  $^{13}\text{C}_6$ -sorbitol as internal standard. The data are available in Suppl. Table 2.

Data were pre-processed by removal of metabolites with more than 75% missing values. For the remaining metabolites a missing value imputation was done using half of the minimum value of the respective metabolite intensity. Contaminating compounds were identified by hierarchical clustering and correlation matrices with a set of known contaminating compounds. Known and yet non-identified contaminants were subsequently

removed from further analysis. A batch effect correction was done by previously published procedures to enable joint analysis of the three independent experiments (Lisec *et al.*, 2011). Outliers were identified by the *grubbs.test* included in the R-package *outliers*. Finally, 76 metabolites were retained for Col-0 and 91 metabolites for N14. Metabolite intensities were divided by the median intensity across all measurements and resulting fold-changes  $\log_2$ -transformed to approximate normal distribution ( $\log_2FC$ ). All presented metabolite data thus represent relative metabolite abundance measures. PCA was executed with the R-package *pcaMethods* including Pareto scaling and centering (Stacklies *et al.*, 2007). Statistical significance of differences in metabolite pool sizes between treatments was tested separately for both accessions for 11 different comparisons (Table 1) by t-test (BH-corrected  $P < 0.05$  (Benjamini & Hochberg, 1995)) in R. The overlap between metabolites with significantly different pool sizes in different comparisons was illustrated by Venn diagrams (R-package *VennDiagram*).

### **Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

For validation of RNA-Seq data the expression of a selection of genes was measured additionally by qRT-PCR as described in detail recently (Sprenger *et al.*, 2018). cDNA was transcribed from 1  $\mu\text{g}$  of the same total RNA previously used for RNA-Seq analysis using SuperScript III Reverse Transcriptase (Thermo Scientific). cDNA yield and quality were tested with primers for the 5' and 3' ends of *GAPDH* (Zuther *et al.*, 2012) by qRT-PCR. Primers for 35 candidate genes were generated using the Primer3 online tool (<http://primer3.wi.mit.edu/>) and checked with blast searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to exclude multiple binding sites. qRT-PCR measurements were done with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) in 384-well plates. Reactions contained a final volume of 5  $\mu\text{L}$ .

containing 2.5  $\mu$ L Power SYBR Green reagent (Applied Biosystems), 0.5  $\mu$ L cDNA (diluted tenfold) and 2  $\mu$ L of 0.5  $\mu$ M primers. For the setup of the plates a pipetting robot Evolution P3 (Perkin Elmer, Zaventem, Belgium) was used. Normalization of the cycle threshold (Ct) values for the candidate genes was done against the mean Ct of four reference genes. The relative gene expression was calculated as  $2^{-\Delta Ct}$  and  $\log_2$ -transformed to obtain approximate normal distribution. All primer sequences including primers for the reference genes are listed in Suppl. Table 3. Normalized expression values for all selected genes are given in Suppl. Table 4. Correlation analysis of RNA-Seq data with expression values from qRT-PCR was performed in R using the *cor* package with Pearson correlation. The correlation plot was generated with *ggplot2* and the plots comparing gene expression from qRT-PCR and RNA-Seq were generated with the R-package *plotrix*.

## RESULTS

### Memory of cold priming improves *Arabidopsis* freezing tolerance

To elucidate if previous cold priming improves the freezing tolerance of plants after a second cold treatment, two *A. thaliana* accessions with intermediate (Col-0) and high freezing tolerance (N14) (Zuther *et al.*, 2012) were investigated. Freezing tolerance was determined after freezing rosettes to different temperatures. After thawing, LT<sub>50</sub> values were calculated from the results of electrolyte leakage measurements. Control plants were investigated after 28 days of growth (C28), after cold priming at 4°C for an additional three days (C28P3), after a subsequent lag phase of seven days at 20°C (C28P3L7) and after a second triggering at 4°C for three days (C28P3L7T3) (Fig. 1). Since the plants showed considerable growth during the seven day lag phase, we also included a developmental control that was initially grown for 35 days (C35) and then exposed to cold priming for three days (C35P3). Freezing tolerance was increased by 2.5°C (Col-0) or 3.1°C (N14) after cold

priming of the younger plants (C28P3 vs C28) and the developmental controls (C35 vs C35P3) showed similar results (Fig. 2). Freezing tolerance decreased to values comparable to both controls (C28 and C35) during the lag phase (C28P3L7). After an additional triggering treatment (C28P3L7T3) freezing tolerance of the rosettes was significantly higher than after priming (C28P3) in both accessions. The  $LT_{50}$  of Col-0 plants reached  $-7.8^{\circ}\text{C}$  after priming and  $-9.1^{\circ}\text{C}$  after triggering, while for N14 the corresponding  $LT_{50}$  values were  $-8.8^{\circ}\text{C}$  and  $-10.6^{\circ}\text{C}$ . Likewise,  $LT_{50}$  values were significantly lower for the triggered plants (C28P3L7T3) than for the primed developmental control plants (C35P3), indicating that increased freezing tolerance was indeed due to a memory effect and not to developmental differences.

### **Treatment effects on transcriptome, lipidome and primary metabolome**

To elucidate the molecular basis of the observed cold memory effect, samples were taken from three independent biological experiments conducted with the accessions Col-0 and N14 at all time points indicated in Fig. 1. The samples were split and aliquots were used for global transcript profiling by RNA-Seq, for lipidomic analysis by UPLC-MS and for the analysis of primary metabolites by GC-MS. For Col-0, 99.5% and for N14 98.0% of all 150 bp single-end Illumina reads could be mapped to the Arabidopsis Col-0 genome sequence. The number of expressed genes that were detected was 24,574 out of a total of 33,602 genes annotated in TAIR10. The lipidomic analysis identified 162 lipid species (Suppl. Table 1), while the GC-MS analysis revealed 76 metabolites for Col-0 and 91 for N14 (Suppl. Table 2).

PCAs were performed with the transcript, lipid and metabolite datasets (Suppl. Fig. 1). They revealed a clear separation of the data from plants exposed to  $20^{\circ}\text{C}$  (C28, C35, C28P3L7) from the data from all plants exposed to  $4^{\circ}\text{C}$  before sampling (C28P3, C35P3, C28P3L7T3) by Principal Component 1 (PC1). In addition, the two accessions were separated by PC2 in the PCA of the transcript data (Suppl. Fig. 1A). Further analysis

indicated that under the different experimental conditions approximately 650 to 750 genes showed significantly different expression levels in N14 compared to Col-0 with an absolute  $\log_2FC$  larger than 1 (Suppl. Table 5). On the other hand, there were almost no significant differences in the abundance of lipids between the accessions (Suppl. Table 5), in agreement with the lack of separation in the PCA. For the primary metabolome, we found significant differences between the accessions that varied strongly with eight to 41 differential metabolites under the different experimental conditions. However, more than half of the significant differences, 77 out of 140 over all conditions, were small and less than  $\log_2FC$  |1| (Suppl. Table 5), explaining the lack of separation of the accessions in the corresponding PCA.

To increase the resolution of the analysis with respect to the different treatments, we subsequently performed PCAs separately with the data from the two accessions. In addition, since we showed earlier that composition of membrane lipids and storage lipids (triacylglycerols, TAGs) respond differently to cold conditions (Degenkolbe *et al.*, 2012), these two datasets were also analyzed separately. The resulting PCAs revealed again a clear separation between warm and cold treated plants. For transcripts this division was driven by PC1, which accounted for 82% and 77% of the total variance in the Col-0 and N14 datasets, respectively (Fig. 3). Similarly, for membrane lipids in N14 and for storage lipids in both accessions, PC1 divided the samples by treatment temperature, contributing between 38% and 71% of the total variance (Fig. 4). Only for membrane lipids in Col-0 this separation was driven by PC2, which contributed only 27% of the total variance. From the distribution of the samples in the scatter plot it is not obvious which factor was underlying PC1, which accounted for 46% of the variance. Data of primary metabolites were again clearly divided by PC1 between warm and cold treated plants from Col-0, where PC1 explained 59% of the total variance. In contrast, PC2 divided warm and cold exposed N14 plants with 37% of the total

variance (Fig. 5). In all these cases there were no clear separations between primed plants (either C28P3 or C35P3) and plants that had experienced a sequence of priming, lag phase and triggering (C28P3L7T3). This indicated that any effects underlying the increased freezing tolerance due to a cold memory were subtle compared to the overall cold response.

### **Developmental effects on transcriptome, lipidome and metabolome**

During the course of our experiments, plants were exposed to three days at 4°C either once or twice. Plants that were cold treated twice were in addition exposed to a lag phase of seven days between the cold treatments. While plant development was arrested during the periods at 4°C, this was not the case during the lag phase. To account for possible developmental effects when comparing primed with primed and triggered plants, we always included developmental controls in our experiments that were grown for an additional seven days at 20°C, labelled C35 and C35P3. These plants should have the same developmental and metabolic characteristics as the plants labelled C28P3L7 and C28P3L7T3, respectively.

The older and younger control plants (C35 vs C28, comparison A; Table 2, Suppl. Table 6) showed no transcriptomic differences in N14 and very few in Col-0. Similarly, for the same comparison more significant differences in lipid composition were detected in Col-0 (40 differences) than in N14 (3) and included primarily lipids with reduced content (37) in the older plants (Table 3, Suppl. Table 7). Primary metabolites showed a similar picture with only few developmental differences overall, but more significant differences in Col-0 (16) than in N14 (4). Overall older plants had mostly reduced metabolite levels, e.g. 13 of 16 metabolites in Col-0 (Table 4, Table 8). Taken together, these data indicate a stronger influence of development on metabolism in Col-0 than N14. This observation is in agreement with the much slower development of N14. N14 did not bolt throughout the experiment

compared to Col-0 that had 0.24 to 0.3 cm long inflorescences at the last four sampling points (Suppl. Fig. 2).

Significant differences between 35 day old and 28 day old primed plants (C35P3 vs. C28P3; comparison E) were also small for transcripts (31 in Col-0 and 1 in N14) and involved mainly genes in Col-0 with a higher expression in the older plants (Table 2). More differences were again observed for lipids and primary metabolites. These differences were more equally distributed between the accessions (Table 3 and 4). In Col-0 91 lipids and 54 metabolites showed developmental differences after priming, with the majority (88 and 42, respectively) of the compounds showing lower content in older plants, similar to the control plants. In N14, on the other hand, more differences of lipid and metabolite content were observed between older and younger plants after priming than in the non-primed control plants. The content of 76 lipids was significantly different between C35P3 and C28P3. Forty seven of these lipids had higher content in older plants, 25 out of 33 metabolites with significantly different abundance were again decreased in older plants. These data indicate that development had a stronger impact on lipid and metabolite content of plants after priming compared to non-primed control plants. These differences between control and primed plants were more pronounced in N14 than in Col-0.

Finally, we compared plants at the end of the lag phase (C28P3L7) with their direct developmental control (C35, comparison G), to validate this control. The analysis showed that there were only three genes that showed a significant difference in expression in this comparison in Col-0, while there were no corresponding genes in N14 (Table 2). Likewise, there were no significant differences in the abundance of any lipids in either accession (Table 3) and only very few metabolites showed significant differences in abundance (four in Col-0 and seven in N14) (Table 4), indicating that our developmental controls were indeed appropriate.

## **The transcriptomic, lipidomic and metabolomic signatures of cold memory**

In agreement with many previous studies, our data indicate a massive effect of cold on the transcriptome, lipidome and metabolome of both accessions. The highest number of genes that showed significant differential expression after cold priming was discovered in 28 day old plants with 2014 (Col-0) and 2101 (N14), compared to 1728 (Col-0) and 1700 (N14) in 35 day old plants (Table 2, comparison B and D). Similarly, comparison B (C28P3 vs C28) revealed 81 and 91 lipids (Table 3) and 61 and 54 metabolites (Table 4) with significant differences in abundance, while comparison D (C35P3 vs C35) revealed 68 and 75 lipids and 62 and 51 metabolites with significant differences in abundance in Col-0 and N14, respectively.

In contrast, the plants showed a much weaker response to the triggering cold treatment than to the priming treatment at the transcriptomic level. After a seven day lag phase and a second cold treatment (C28P3L7T3 vs C28P3L7, comparison J) only 740 (Col-0) and 996 (N14) genes showed a significant cold response (Table 2). These were only one third or half of the significant changes detected after priming in the two accessions. However, this picture was completely different at the lipidomic and metabolomic levels, where comparison J revealed 78 and 70 significant differences in lipid and 59 and 43 in metabolite abundance in Col-0 and N14, respectively (Table 3 and 4). These numbers were very similar to those determined in comparisons B and D that were described above.

To identify genes, lipids and metabolites uniquely associated with cold memory, we used two complementary strategies. One was to directly compare data obtained from plants after triggering (C28P3L7T3) with their developmental control (C35P3, comparison K). In the second approach we identified transcripts, lipids and metabolites that showed significant changes only after triggering compared to the control samples (C28P3L7T3 vs C28,

comparison H), but not after priming compared to the same control (C28P3 vs C28, comparison B; C35P3 vs C28, comparison C). Comparing all cold treatments to the same reference point eliminates the additional variability that may be otherwise introduced by using two different developmental references.

A direct comparison of triggered samples with the primed developmental control (C28P3L7T3 vs C35P3, comparison K) resulted only in one significantly differentially expressed gene in Col-0 and N14, each, which showed very large induction (Table 2). In Col-0 a gene encoding a protein with a domain of unknown function (AT5G28810) showed a  $\log_2$ FC of 36.8 and in N14 a gene encoding an auxin efflux carrier family protein (AT5G65980) showed a  $\log_2$ FC of 28.9 (Suppl. Table 6).

For the identification of unique significant expression changes after triggering, the overlap of the results from comparisons B (C28P3/C28), H (C28P3L7T3/C28) and C (C35P3/C28) was analysed and filtered for unique genes in comparison H to exclude expression changes caused by priming or development (Fig. 6, Suppl. Table 6). This analysis identified 93 genes (54 up-, 39 down-regulated) in Col-0 and 128 genes (49 up-, 79 down-regulated) in N14. To identify functional groups of genes that respond to triggering conditions, an over-/underrepresentation analysis of genes identified in comparisons B, H and C was performed using the MapMan bin structure. This analysis measures statistically whether a particular functional group (bin) of genes contains more up- or down-regulated genes than expected from an equal distribution among all bins. Genes identified in comparison H (after triggering) in Col-0 were uniquely overrepresented in functional categories such as lipid metabolism, secondary metabolism and stress (Fig. 7). In N14 no unique overrepresented bin was found among upregulated genes for comparison H (Fig. 7), but the abiotic stress bins, heat and unspecified contained more down-regulated genes than expected (Fig. 8).

In agreement with the overrepresentation of uniquely upregulated genes from comparison H in the lipid metabolism bin, we also found 25 lipids in Col-0 and 33 in N14 with an overlap of 19 that were significantly increased in abundance after triggering compared to the primed developmental control (comparison K, Table 3, Suppl. Table 7). These lipids included, in Col-0 and N14 respectively, nine and five digalactosyldiacylglycerols (DGDG), six and eight monogalactosyldiacylglycerols (MGDG), four lyso-phosphatidylcholines (lysoPC), one sulfoquinovosyldiacylglycerol (SQDG) and four and sixteen TAGs. However, all these changes were rather moderate, with only LysoPC 18:1 reaching a  $\log_2FC > 1$  (Suppl. Table 7).

Using the same approach as described above for transcripts, we also identified unique lipids that were significantly changed in abundance after triggering using the comparisons B (C28P3/C28), H (C28P3L7T3/C28) and C (C35P3/C28) (Fig. 9, Suppl. Table 7). All lipids identified as unique in comparison H showed an increased abundance after triggering, including DGDG 34:1, PC 32:1\_c and SQDG 36:6 in Col-0 and DGDG 34:1, DGDG 34:4\_a, MGDG 34:1 as well as the three arabidopsides A\_MGDG OPDA/dnOPDA, B\_MGDG OPDA/OPDA and D\_DGDG OPDA/OPDA in N14. Only the three arabidopsides had a  $\log_2FC$  above 1 (Suppl. Table 7). While the increases in the arabidopsides were only significant in N14, similar increases were also evident in Col-0 (Suppl. Table 8).

Differences in metabolite content between triggered plants and the primed developmental control (comparison H) were highly accession specific, since there were no significant differences in N14, but 41 in Col-0 (Table 4). Of particular interest were some of the metabolites with a  $\log_2FC > 1$ , including the well-known cold-induced compatible solutes proline, sucrose and raffinose, and the raffinose precursor galactinol, together with two other amino acids, four organic acids, erythritol and six unknown analytes (Suppl. Table 9).

Filtering metabolite differences in the comparisons B, H and C for those unique in H revealed

only ornithine in N14, but no metabolites in Col-0 (Fig. 10). It is important to note that due to the choice of GC-MS based profiling ornithine represents the sum of urea cycle intermediates, namely arginine, citrulline and ornithine.

### **Validation of RNA-Seq data by qRT-PCR**

We selected a set of 35 genes from the RNA-Seq dataset that either showed a triggering-specific expression pattern in at least one of the accessions or are well-known cold induced genes. Correlation analysis confirmed the good correspondence of the expression values obtained from the two methods (Suppl. Fig. 3a). To illustrate this correspondence in more detail we have also plotted the expression patterns obtained with both methods of six of these candidate genes. Panels (b) and (c) show genes with a significant triggering response in Col-0, while panels (d) and (e) show two genes with a significant triggering response in N14. The data in (e) also illustrate that not only the relative expression levels, but also the variance in the data determine whether a significant triggering response relative to the priming response can be determined. The corresponding gene only showed a significant triggering response in N14, although it is also clearly induced in Col-0. Finally, panels (f) and (g) illustrate the behavior of two typical "classical" cold regulated genes, *COR15A* and *LTI78/RD29A*. As described above, both genes showed consistent cold induction under all cold treatments, which was detectable by both RNA-Seq and qRT-PCR.

## **DISCUSSION**

### **Cold priming and memory improve Arabidopsis freezing tolerance**

To investigate the effect of a triggering response after cold priming on the freezing tolerance of two Arabidopsis accessions, an initial growth phase of four weeks was combined with three days of priming and triggering, each, and an intervening lag phase of seven days.

To account for the long lag phase, an additional developmental control was included, with an initial growth phase of five weeks. The  $LT_{50}$  values for the C28 and C35 plants (-5.28 and -6.20°C for Col-0; -5.67 and -6.87°C for N14) were similar to values reported for 42 day old plants of the two accessions under the same conditions (-5.34°C, -7.35°C) (Zuther *et al.*, 2012). After three days of cold priming the  $LT_{50}$  reached -7.76°C (C28P3) and -7.25°C (C35P3) in Col-0 and -8.79°C and -9.34°C in N14. These values were substantially higher than  $LT_{50}$  values obtained after two weeks of cold treatment (-9.68°C in Col-0 and -12.10°C in N14) (Zuther *et al.*, 2012). This is in agreement with the fact that Arabidopsis plants need at least seven days to reach their full freezing tolerance at 4°C (Wanner & Junttila, 1999).

A seven day lag phase was sufficient to reduce the freezing tolerance of primed plants back to levels comparable to the control plants, while a three day lag phase after two weeks of cold treatment was insufficient (Zuther *et al.*, 2015). This is comparable with memory after short priming to drought which was also lost after seven days (Ding, Fromm & Avramova, 2012). One of the possible responses of a primed plant to a triggering stimulus is an increased intensity of the reaction (Hilker *et al.*, 2016). This mode of priming is also what we observed when we compared the freezing tolerance of primed with primed and triggered plants. To our knowledge this is the first report of an adaptive cold memory in plants leading to higher freezing tolerance. A stronger stress response after triggering of previously primed plants has been described in Arabidopsis under salt (Sani, Herzyk, Perrella, Colot & Amtmann, 2013), drought (Ding *et al.*, 2012) and heat stress (Sedaghatmehr, Mueller-Roeber & Balazadeh, 2016, Stief *et al.*, 2014), indicating that adaptive stress memory may be a general response mechanism for Arabidopsis when confronted with abiotic stresses.

### **Unique transcriptomic responses to a triggering cold stimulus**

Transcriptomic, lipidomic and metabolomic changes during cold exposure of

Arabidopsis plants have been described in detail before (see Chinnusamy *et al.*, 2007, Guy, Kaplan, Kopka, Selbig & Hinch, 2008, Hinch *et al.*, 2012, Thomashow, 2010 for reviews).

We will therefore not discuss general cold responses, but rather focus on aspects that are directly related to cold memory. In general, however, our findings on cold responses are in agreement with those reported in the literature and all detailed comparisons can be found in the supplemental data section. In addition, validation of a subset of RNA-Seq data by qRT-PCR showed a good correlation between the two methods and confirmed the cold induction of well-known genes such as *COR15A*.

Globally, plants showed a weaker transcriptomic response after triggering (comparison J, 740/996 significant changes in Col-0/N14) than after priming (comparison B, 2014/2101 significant changes in Col-0/N14). Since the sampling was done after three days of cold treatment the mode of priming cannot be unambiguously assigned. We cannot exclude that an early response to the second stress had the same intensity but may have decreased faster. In part, however, this weaker response seemed to be due to a developmental difference, as cold priming of the older plants also resulted in a slightly reduced transcriptomic response (comparison D, 1728/1700 significant changes in Col-0/N14). The further reduction in the transcriptomic response to cold during triggering may be due to active regulation. This has been described for dehydration stress where drought-responsive genes that did not respond to a second drought stress were named “revised-response” memory genes (Liu, Ding, Fromm & Avramova, 2014). During the first stress the response seems to be comprehensive to prepare the plants for even harsher conditions whereas after a second stress treatment of the same strength the full response is not elicited anymore. Nevertheless, there was a considerable overlap of genes that were regulated during priming (comparison B) and during triggering (comparison J) (Suppl. Table 4). These included well-known cold induced genes encoding

COR proteins such as LTI78, COR15A, COR15B, XERO2 and KIN1. In addition, we also found a number of genes that were uniquely regulated during triggering and thus constitute potential memory genes, i.e. genes with a specific function in cold memory. Obviously, all such candidate genes will require experimental validation, e.g. through mutant or overexpressor studies.

● Only two genes were highly up-regulated after triggering compared to the developmental control (comparison K). In Col-0 this was a gene encoding a protein with a domain of unknown function (AT5G28810) and in N14 a gene encoding an auxin efflux carrier family protein (AT5G65980). The former protein (AT5G28810) is also annotated as homolog of an ubiquitin-like-specific protease 1, which functions in the SUMOylation pathway in yeast. It processes full-length suppressor of mif two 3 (SMT3) to its mature form and deconjugates SMT3 from its target proteins (Li & Hochstrasser, 1999). The auxin efflux carrier family protein belongs to the PIN-LIKES (PILS) family of auxin transport facilitators (Barbez *et al.*, 2012), has auxin:proton symporter activity and is involved in polar auxin transport. PILS proteins are involved in the regulation of intracellular auxin accumulation at the endoplasmatic reticulum, which determines the availability of the hormone for nuclear auxin signalling. By determining the cellular sensitivity to auxin they are needed for auxin-dependent regulation of plant growth (Barbez *et al.*, 2012).

The unique transcriptomic triggering responses identified from the contrast of comparisons B, C and H were largely specific for the accessions, since of 93 and 128 transcripts with significantly changed abundance in Col-0 and N14, respectively, only ten were common. The expression of these overlapping genes changed in the same direction in both accessions. One possible explanation for the small overlap of triggering-specific genes between the accessions could be that some of the genes induced uniquely after triggering in one accession were already up-regulated in the other accession at the end of the lag phase

(comparison G). However, this was not the case since only two such genes were identified in Col-0 and none in N14. Nevertheless, one of the most highly induced genes unique in comparison H ( $\log_2FC = 6.5$ ), encoding a protein of unknown function (AT2G44240), showed significantly higher expression in N14 compared to Col-0 at the end of the memory phase (C28P3L7;  $\log_2FC = 3.9$ ) and in the younger control (C28;  $\log_2FC = 6.5$ ). This gene may be an interesting candidate for a pre-adaptation in a more freezing tolerant accession.

Uniquely up-regulated transcripts after triggering in Col-0 were overrepresented in functional bins related to lipid metabolism, secondary metabolism and stress, whereas one bin was overrepresented for uniquely down-regulated transcripts in Col-0 and two stress related bins in N14. Differences in cold response between accessions were described before for lipid abundance (Degenkolbe *et al.*, 2012), flavonol, anthocyanin (Korn, Peterek, Mock, Heyer & Hinch, 2008, Schulz, Tohge, Zuther, Fernie & Hinch, 2015) and primary metabolite content (Hannah *et al.*, 2006, Zuther *et al.*, 2012). In addition, correlations between the expression of cold responsive genes or genes involved in flavonol and anthocyanin biosynthesis and freezing tolerance were detected (Hannah *et al.*, 2006, Schulz *et al.*, 2015, Zuther *et al.*, 2012). A unique induction of the respective genes in Col-0 during triggering may indicate a functional cold adaptation of metabolism, whereas N14 may less rapidly quench cold memory and may already be better prepared for a second cold trigger at the end of the lag phase. We will discuss this hypothesis in more detail for primary metabolites below.

In N14 additionally to the highly up-regulated gene encoding an auxin efflux carrier family protein, several uniquely up-regulated transcripts were related to growth processes, such as the highly ABA-induced PP2C gene 2 (AT1G07430) involved in the positive regulation of a gibberellic acid mediated signaling pathway, gibberellin 2-oxidase 1 (AT1G78440) involved in gibberellin metabolism, MIR159/MIR159A (AT1G73687)

encoding a miRNA involved in regulation of vegetative phase change, with a double mutant for MIR159A and B showing reduced growth and SPIRAL1-like5 (AT4G23496) involved in regulation of cortical microtubule organization (Suppl. Table 6). Similar transcriptional responses as noted above were also observed in transcriptional memory of dehydration stress in Arabidopsis, including altered gene expression that points towards increased synthesis of protective and detoxifying metabolites, coordination of growth, re-adjustment of osmotic equilibrium and re-adjusting interactions between stress and hormone regulated pathways (Ding *et al.*, 2013).

### **Arabidopsides as unique signature molecules of cold memory**

During cold priming comprehensive changes in lipid composition have been reported, but no priming-specific lipids could be identified (Barrero-Sicilia, Silvestre, Haslam & Michaelson, 2017, Degenkolbe *et al.*, 2012, Lynch & Steponkus, 1987, Palta, Whitaker & Weiss, 1993, Tarazona, Feussner & Feussner, 2015, Uemura, Joseph & Steponkus, 1995, Uemura & Steponkus, 1994, Uemura & Yoshida, 1984). Also, an increase in the content of highly unsaturated fatty acids in membrane lipids has been reported (Wang, Li, Li & Welti, 2006, Welti *et al.*, 2002). In addition, a massive increase in the amount of TAGs was found after 14 days of cold priming in a set of 15 Arabidopsis accessions that was attributed to an excess of fixed carbon due to strongly reduced growth rates in the cold (Degenkolbe *et al.*, 2012). An increase in the content of several highly unsaturated TAGs during triggering in both accessions (comparison K) might be caused by the same metabolic reasons. Two MGDG species (34:2\_a and 34:3) with an increased content after triggering in N14 showed a positive correlation with acclimated freezing tolerance across several accessions in an earlier study (Degenkolbe *et al.*, 2012). Only one lipid, lysoPC (18:1), showed a  $\log_2FC > 1$  in both accessions after triggering compared to the primed developmental control (comparison K).

Lysophospholipids are minor membrane components and act as mediators of signaling, e.g. through release into the extracellular space where they may interact with receptors (Hou, Ufer & Bartels, 2016). They accumulate in response to freezing, wounding, elicitor application or infection with pathogens (Lee *et al.*, 1997, Narvaez-Vasquez, Florin-Christensen & Ryan, 1999, Scherer, 2002, Viehweger, Dordschbal & Roos, 2002, Welti *et al.*, 2002, Wi, Seo, Cho, Nam & Park, 2014). Whether the lysoPC identified here plays a signaling role in cold memory remains to be established.

A striking finding was the significant increase in the content of three arabidopsides in N14 after triggering, but not after priming (Suppl. Table 8). This response was also detected in Col-0 with similar  $\log_2FC$ , although the differences were not significant. This increased arabidopside content could either be caused by a higher accumulation during the three days of triggering or by a higher content already at the end of the lag phase. Since no significant changes were detected for either accession at the end of the lag phase in comparison F (C28P3L7/C28) we conclude that the observed changes were induced by triggering.

Arabidopsides contain two oxylipin chains and the ones increased after triggering are denoted as A (MGDG OPDA/dnOPDA), B (MGDG OPDA/OPDA) and D (DGDG OPDA/OPDA) (Hisamatsu *et al.*, 2005). They are oxidatively modified membrane lipids containing either 12-oxophytodienoic acid (OPDA) or dinor-oxophytodienoic acid (dnOPDA) (Andersson, Kourtchenko, Dangl, Mackey & Ellerstrom, 2006, Buseman *et al.*, 2006). OPDA and dnOPDA form, together with jasmonic acid (JA) and other cyclic oxylipins, the JA group of compounds (Mosblech, Feussner & Heilmann, 2009, Pohl & Kock, 2014, Savchenko *et al.*, 2014). They are well known as signaling molecules involved in developmental and stress responses (see Hou *et al.*, 2016 for a review). Arabidopsides are synthesized in several steps from  $\alpha$ -linolenic acid esterified in galactolipids of chloroplast membranes (Wasternack, 2014). None of the genes encoding enzymes involved in this

pathway showed a significant up-regulation during triggering, indicating a specific post-transcriptional or metabolic regulation. Alternatively, these genes may have been only transiently induced during triggering and had already returned back to control levels after three days in the cold.

Arabidopsides are only found in a limited number of species of the genus *Arabidopsis* (Böttcher & Weiler, 2007) and might function as a storage form of signaling molecules that are rapidly releasable upon stress (Mosblech *et al.*, 2009). They are accumulated in response to wounding, darkness, osmotic stress and as defense response to herbivory. They have growth inhibiting effects on bacterial and fungal pathogens and promote senescence (Andersson *et al.*, 2006, Buseman *et al.*, 2006, Glauser *et al.*, 2008, Hisamatsu, Goto, Hasegawa & Shigemori, 2006, Kourtchenko *et al.*, 2007, Maeda, Sage, Isaac, Welti & Dellapenna, 2008, Schafer, Fischer, Baldwin & Meldau, 2011, Vu *et al.*, 2012, Xiao *et al.*, 2010, Zoeller *et al.*, 2012). Most of these functions are mediated by the release of *cis*-OPDA as an immediate precursor of JA from arabidopsides by lipase activity (Buseman *et al.*, 2006, Dave & Graham, 2012). For *dn*OPDA no signaling function has been reported yet. Oxylipins form a small fraction of the total membrane lipids and have a fast turnover. More than 150 genes respond to the application of *cis*-OPDA but not to JA or methyl JA in *Arabidopsis* (Taki *et al.*, 2005). Not much is known about the molecular mechanisms of OPDA signaling, but transcription factors involved in abiotic and biotic stress tolerance are induced together with genes related to detoxification, stress responses and secondary metabolism when leaves are treated with OPDA (Mueller *et al.*, 2008, Taki *et al.*, 2005).

The active form of JA, JA-isoleucine, synthesized from OPDA, promotes the interaction of JA ZIM domain (JAZ) transcription factors and the JA receptor F-box ubiquitin ligase Coronatine Insensitive1 (COI1), initiating the degradation of JAZ proteins followed by induction of a set of JA-dependent genes which depend on the MYC2 transcription factor

(Dave & Graham, 2012, Hou *et al.*, 2016). JA is also an important positive regulator of the ICE1-CBF transcriptional pathway in Arabidopsis involved in the regulation of freezing tolerance (Hu, Jiang, Wang & Yu, 2013, Hu *et al.*, 2017). Cold treatment induces endogenous JA production through the induction of genes encoding enzymes involved in JA biosynthesis. Prolonged cold exposure also increases JA and salicylic acid (SA) levels in wheat contributing to improved freezing tolerance (Kosova *et al.*, 2012). Repeated exposure to dehydration leads to a reduced transcriptomic response of several genes that respond to the first dehydration stress. These genes are mainly involved in JA biosynthesis, JA-signaling and JA-mediated stress responses (Liu & Avramova, 2016, Liu, Staswick & Avramova, 2016). The authors proposed that increased JA synthesis might be responsible for the lack of drought induction of downstream genes and that this may be part of the observed stress memory. A possible role of OPDAs in this process has not been reported yet.

There is also evidence that OPDA can regulate gene expression independent of JA signaling after wounding and dehydration via COI1 (Ribot, Zimmerli, Farmer, Reymond & Poirier, 2008) or after biotic stress in a COI1-independent manner (Stintzi, Weber, Reymond, Browse & Farmer, 2001, Stotz, Mueller, Zoeller, Mueller & Berger, 2013, Taki *et al.*, 2005). Interestingly, *COI1* was recently implicated in freezing tolerance in a genome wide association study with 499 worldwide accessions (Horton, Willems, Sasaki, Koornneef & Nordborg, 2016).

### **Col-0 and N14 have different metabolic responses during triggering**

Since the finding that the ratio of TCA cycle metabolites acts as critical determinant of the activity of enzyme families controlling epigenetic modifications and can therefore influence innate immune memory in invertebrates (Netea *et al.*, 2016), metabolites in plants might also be considered as regulators of memory processes. During cold acclimation a

major remodeling of the metabolome occurs including the accumulation of compatible solutes such as sugars and certain amino acids (for a review see Guy *et al.*, 2008). Freezing tolerance of *Arabidopsis* accessions and their crosses could be predicted from their metabolite composition (Korn *et al.*, 2010), but a functional role has not been established for any single metabolite.

The search for unique metabolic changes after triggering identified from the contrast of comparisons B, C and H yielded only significantly reduced ornithine content in N14, but none in Col-0. The non-protein amino acid ornithine represents in our current analysis the sum of urea cycle intermediates. The urea cycle is an important source of arginine and linked to the glutamic acid pool. Likewise, glutamic acid is an important precursor of glutamate and proline biosynthesis (Kalamaki, Merkouropoulos & Kanellis, 2009). Proline has been recognized as an important compatible solute under cold and other stress conditions and was massively accumulated in our experiments both during cold priming and triggering. In addition, we also found a strong up-regulation of a gene encoding the key proline biosynthesis enzyme  $\Delta$ -1-pyrroline-5-carboxylate synthase (AT3G55610) under both conditions. Ornithine was discussed in this context as a non-toxic source for a fast conversion into proline, which is toxic in high concentrations under non-stress conditions (Maggio *et al.*, 2002). In addition to its function as a compatible solute, proline can act as a metabolic signal for the regulation of redox homeostasis (Szabados & Savoure, 2010).

Whereas no uniquely changed metabolite was found in Col-0 after triggering, a significant difference in the content of 41 metabolites was specifically detected in Col-0 after comparison of the triggered plants (C28P3L7T3) with the primed developmental control (C35P3, comparison K). Interestingly, this list contained all members of the raffinose biosynthetic pathway, namely raffinose, sucrose, the galactose donor galactinol and the galactinol precursor *myo*-inositol. Raffinose and sucrose content after cold priming are

correlated with the freezing tolerance of a collection of 54 *Arabidopsis* accessions (Zuther *et al.*, 2012). However, the accumulation of raffinose in *Galactinol Synthase* overexpressing lines or the lack of cold induced accumulation of raffinose in *Raffinose Synthase* knock-out lines did not impact *Arabidopsis* freezing tolerance measured with an electrolyte leakage assay (Zuther *et al.*, 2004). However, raffinose is not only accumulated in the cytosol, but also in the chloroplast stroma during cold priming (Nägele & Heyer, 2013) and functions there by specifically stabilising photosystem II during freezing (Knaupp, Mishra, Nedbal & Heyer, 2011), a function we would not detect in the electrolyte leakage assay.

At the end of the lag phase (C28P3L7) Col-0 showed four metabolites with significantly changed content compared to the developmental control (C35, comparison G) and N14 seven. Only in N14 the content of sucrose, raffinose, galactinol and ornithine was increased in this comparison, suggesting that this accession was able to retain a larger fraction of these metabolites during the lag phase and maintain the memory to remain prepared for a future stress event. This is in agreement with a slower decline of sucrose and raffinose content in N14 compared to Col-0 during a three day lag phase under identical conditions (Zuther *et al.*, 2015). Furthermore, the increase in raffinose content during triggering (C28P3L7T3/C28P3L7) was smaller in N14 than in Col-0 (comparison J,  $\log_2FC$  5.8 vs. 6.8), while during priming (C28P3 vs C28 and C35P3 vs C35) the increase in raffinose content was higher in N14 (see also Zuther *et al.*, 2012; Zuther *et al.*, 2016 for cold acclimation). While both accessions stem from low-elevation areas, the origin of N14 is further North than that of Col-0, in agreement with the higher acclimated freezing tolerance of the Russian accession (Zuther *et al.*, 2012). Unfortunately, there is no weather data of sufficiently high spatial and temporal resolution available to judge whether conditions triggering a memory response would be more prevalent in the habitats of either of the accessions.

## CONCLUSIONS

We could show for the first time that plants are able to remember a cold priming event, thus improving their freezing tolerance after a subsequent triggering cold stress. The response to cold priming is memorized over a period of at least seven days in Arabidopsis, which is considerable given the short life cycle of this species. Our data clearly show that the increased freezing tolerance after triggering is not a result of plant development during the seven-day lag phase.

Superior freezing tolerance after cold triggering was associated with the induction of a new transcriptomic response rather than a stronger expression of cold priming responsive genes. The annotation of memory-specific genes pointed to an activation of lipid metabolism, secondary metabolism and stress responses in Col-0 and mainly growth related functions in N14. The latter finding is in agreement with the high induction of a gene encoding an auxin efflux carrier family protein during triggering in N14. Lipidomic analysis confirmed the influence of cold triggering on lipid metabolism and identified three arabidopsides as potential mediators of cold stress memory in both accessions. Raffinose and its biosynthetic precursors were more strongly accumulated in Col-0 during triggering than in N14, which retained a larger part of these metabolites that were accumulated during cold priming at the end of the lag phase. This finding indicates that metabolism may be involved in maintaining cold memory. For an overview of the reported memory and triggering responses in the two accessions see Fig. 11. It is tempting to speculate that the more freezing tolerant accession N14 with a more Northern origin is able to stay more prepared for repeated cold events and maintains cold memory longer than the less freezing tolerant accession Col-0. This study confirms the hypothesis of a transcriptional stress memory that was based on dehydration stress experiments (Ding *et al.*, 2012, Ding *et al.*, 2013). However, it adds further potential molecular determinants of stress memory, such as lipids and metabolites to the response

pattern. Our analyses confirm that the triggering response is distinguishable from the priming response as was previously shown for thermo-priming at high temperatures (Bäurle, 2016).

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Accepted Article

**Table 1:** Comparisons performed for DGE, metabolite and lipid analysis with both accessions.

Com- parison	Description	Detail	Effect
A	C35/C28	Developmental control vs. control	Developmental (warm)
B	C28P3/C28	Primed vs. control	Priming
C	C35P3/C28	Developmental control primed vs. control	Priming including development
D	C35P3/C35	Primed vs. developmental control	Priming
E	C35P3/C28P3	Developmental control primed vs. primed	Developmental (cold)
F	C28P3L7/C28	Memory vs. control	Memory (warm)
G	C28P3L7/C35	Memory vs. developmental control	Developmental control for memory
H	C28P3L7T3/C28	Triggered vs. control	Triggering including development
I	C28P3L7T3/C28P3	Triggered vs. primed	Memory (cold)
J	C28P3L7T3/C28P3L7	Triggered vs. memory	Triggering
K	C28P3L7T3/C35P3	Triggered vs. developmental control	Developmental control for triggering

**Table 2:** DGE analysis of different comparisons of control/control, primed/control and primed/primed and triggered/control or triggered/primed samples (see Table 1) for Col-0 and N14. DGE was determined for  $FDR < 0.1$  and an absolute  $\log_2FC > 1$ . The number of identical transcripts in Col-0 and N14 for each comparison is shown as overlap.

Comparison	Detail	Col-0			N14			Overlap		
		Up	Down	Total	Up	Down	Total	Up	Down	Total
A	C35/C28	32	1	33	0	0	0	0	0	0
B	C28P3/C28	794	1220	2014	821	1280	2101	560	904	1464
C	C35P3/C28	618	817	1435	582	967	1549	355	561	916
D	C35P3/C35	706	1022	1728	587	1113	1700	416	710	1126
E	C35P3/C28P3	30	1	31	0	1	1	0	0	0
F	C28P3L7/C28	27	1	28	0	0	0	0	0	0
G	C28P3L7/C35	2	1	3	0	0	0	0	0	0
H	C28P3L7T3/C28	520	591	1111	482	823	1305	286	399	685
I	C28P3L7T3/C28P3	68	2	70	0	0	0	0	0	0
J	C28P3L7T3/C28P3L7	264	476	740	345	651	996	180	311	491
K	C28P3L7T3/C35P3	1	0	1	1	0	1	0	0	0

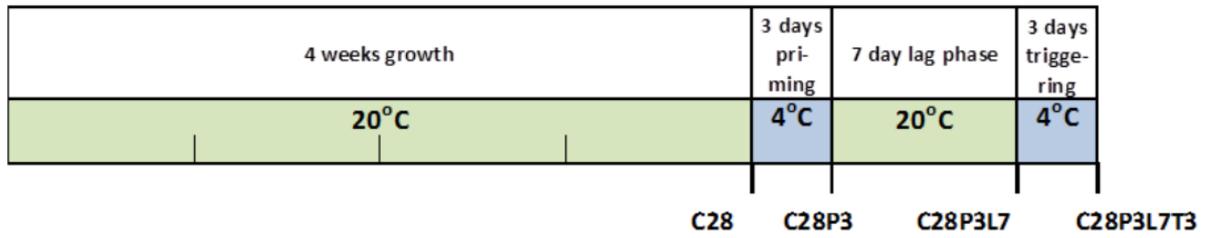
**Table 3:** Significantly changed lipids for different comparisons of control/control, primed/control and primed/primed and triggered/control or triggered/primed samples (see Table 1) for Col-0 and N14. The number of identical lipids in Col-0 and N14 for each comparison is shown as overlap.

Comparison	Detail	Col-0			N14			Overlap		
		Up	Down	Total	Up	Down	Total	Up	Down	Total
A	C35/C28	3	37	40	2	1	3	1	1	2
B	C28P3/C28	65	16	81	65	26	91	62	16	78
C	C35P3/C28	36	35	71	44	37	81	33	34	67
D	C35P3/C35	53	15	68	43	32	75	36	15	51
E	C35P3/C28P3	3	88	91	47	29	76	0	27	27
F	C28P3L7/C28	5	31	36	4	12	16	2	12	14
G	C28P3L7/C35	0	0	0	0	0	0	0	0	0
H	C28P3L7T3/C28	47	14	61	56	25	81	45	13	58
I	C28P3L7T3/C28P3	3	25	28	1	23	24	0	15	15
J	C28P3L7T3/C28P3L7	73	5	78	55	15	70	47	4	51
K	C28P3L7T3/C35P3	25	0	25	33	0	33	19	0	19

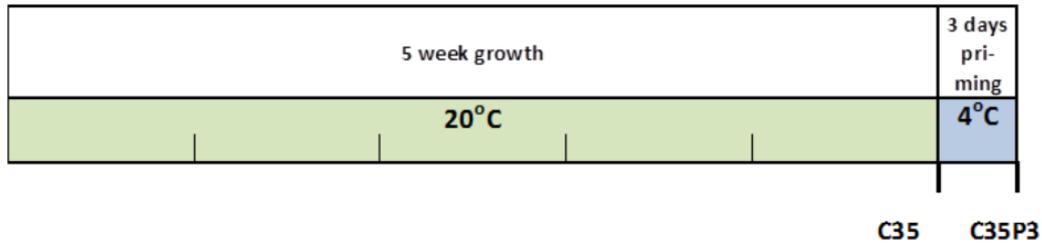
**Table 4:** Significantly changed metabolites for different comparisons of control/control, primed/control and primed/primed and triggered/control or triggered/primed samples (see Table 1) for Col-0 and N14. The number of identical metabolites in Col-0 and N14 for each comparison is shown as overlap.

Comparison	Detail	Col-0			N14			Overlap		
		Up	Down	Total	Up	Down	Total	Up	Down	Total
A	C35/C28	3	13	16	3	1	4	1	0	1
B	C28P3/C28	53	8	61	54	8	62	38	5	43
C	C35P3/C28	57	11	68	49	5	54	36	1	37
D	C35P3/C35	56	6	62	52	6	58	39	1	40
E	C35P3/C28P3	12	42	54	10	29	39	2	15	17
F	C28P3L7/C28	12	6	18	11	3	14	5	1	6
G	C28P3L7/C35	4	0	4	7	0	7	1	0	1
H	C28P3L7T3/C28	51	1	52	45	4	49	32	0	32
I	C28P3L7T3/C28P3	9	29	38	13	22	35	4	10	14
J	C28P3L7T3/C28P3L7	57	2	59	47	4	51	35	1	36
K	C28P3L7T3/C35P3	28	13	41	0	0	0	0	0	0

### Priming and Triggering Setup

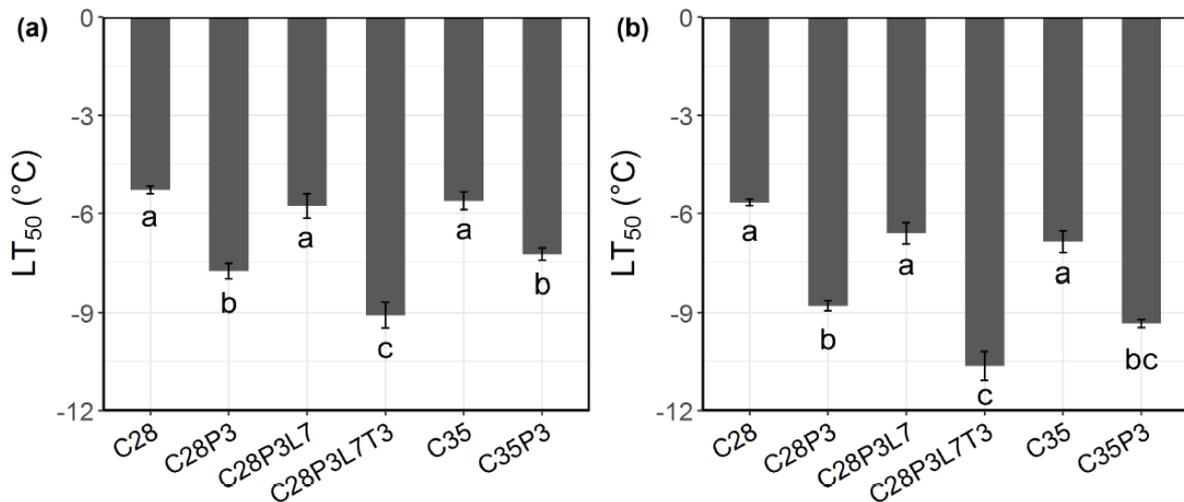


### Developmental Control Setup

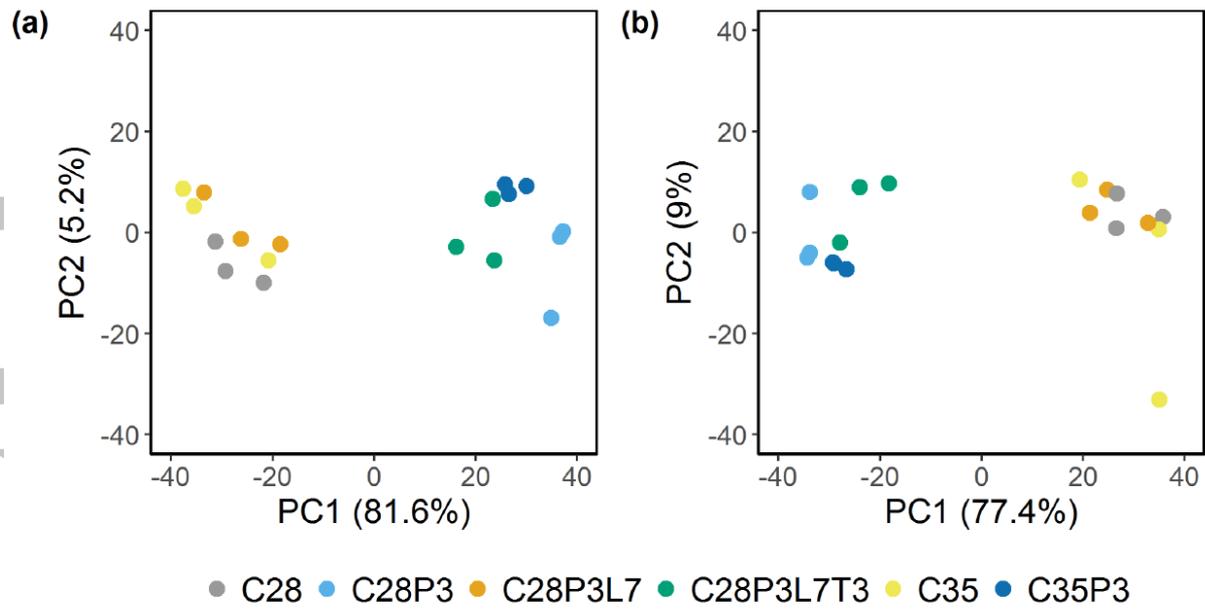


**Figure 1:** Experimental setup for the priming and triggering experiment. C28 and C35 indicate the sampling time points of the control plants, C28P3 and C35P3 the sampling time points of the respective cold primed plants, C28P3L7 the sampling time point of cold primed plants after a seven day lag phase at 20°C, and C28P3L7T3 the sampling time point of plants that were exposed to a second cold triggering treatment.

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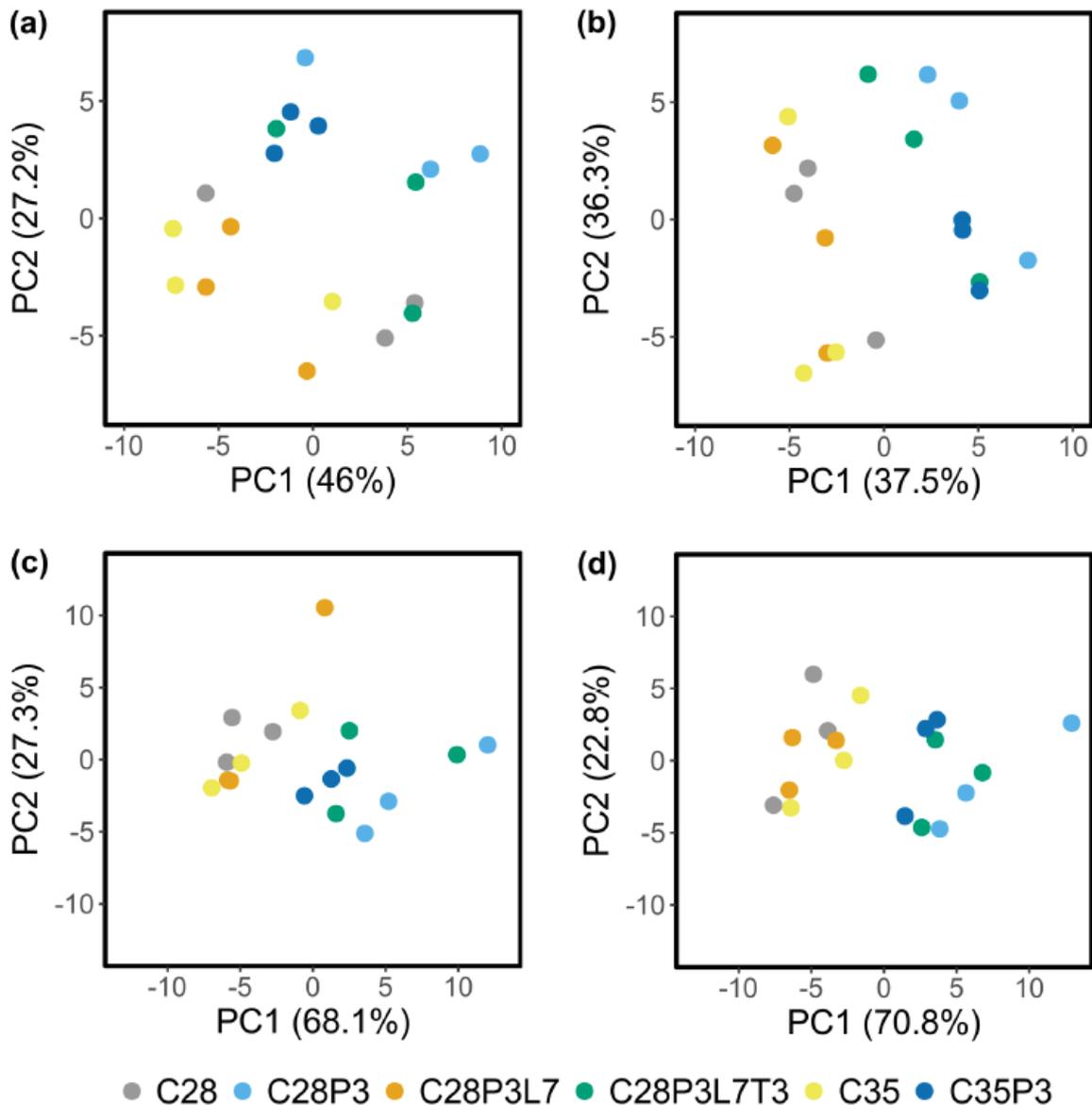


**Figure 2:** Freezing tolerance of rosettes of the *Arabidopsis* accessions Col-0 (a) and N14 (b) after 28 days of growth under control conditions (C28), after cold priming at 4°C for three days (C28P3), after a lag phase of seven days at 20°C (C28P3L7) and after triggering at 4°C for three days (C28P3L7T3). Additionally, LT<sub>50</sub> values for 35 days old plants under control (C35) and primed conditions are shown (C35P3) as developmental controls. Freezing tolerance was measured with an electrolyte leakage assay and is expressed as LT<sub>50</sub>, the temperature which resulted in 50% ion leakage from the leaves. The bars represent the mean ± SEM of three independent experiments with five biological replicates each. Significance of differences in LT<sub>50</sub> among the treatments were evaluated separately for Col-0 and N14 by ANOVA. Different letters indicate significant differences at  $p < 0.05$ .

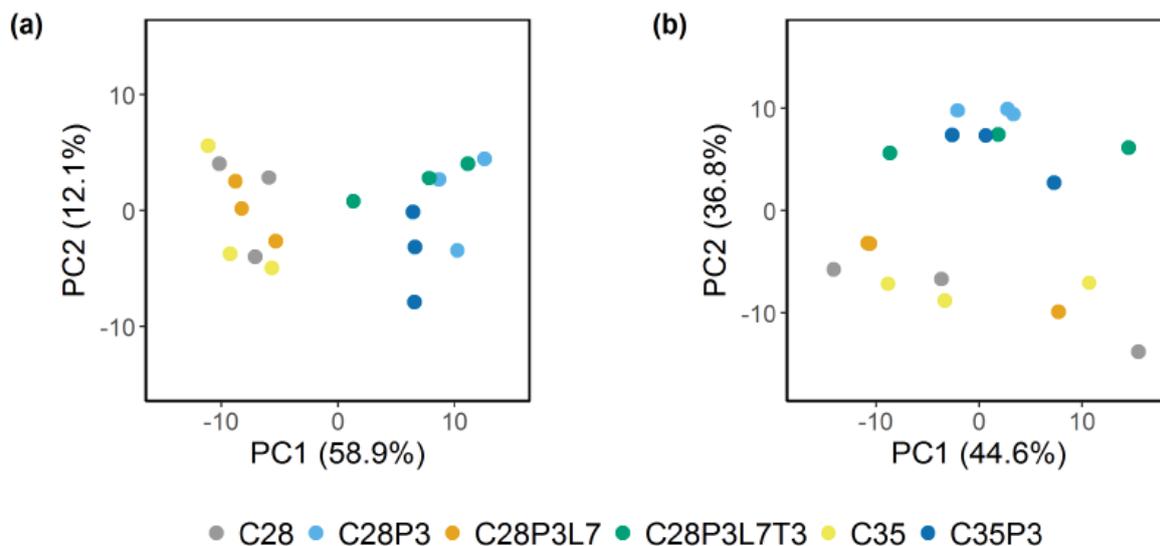


**Figure 3:** Principal Component Analysis (PCA) of RNA-Seq data showing Principal Component (PC) 1 and PC2 for the accessions Col-0 (a) and N14 (b) at the indicated conditions. Shown are the scores of regularized log transformed (rlog) data as means of three independent experiments.

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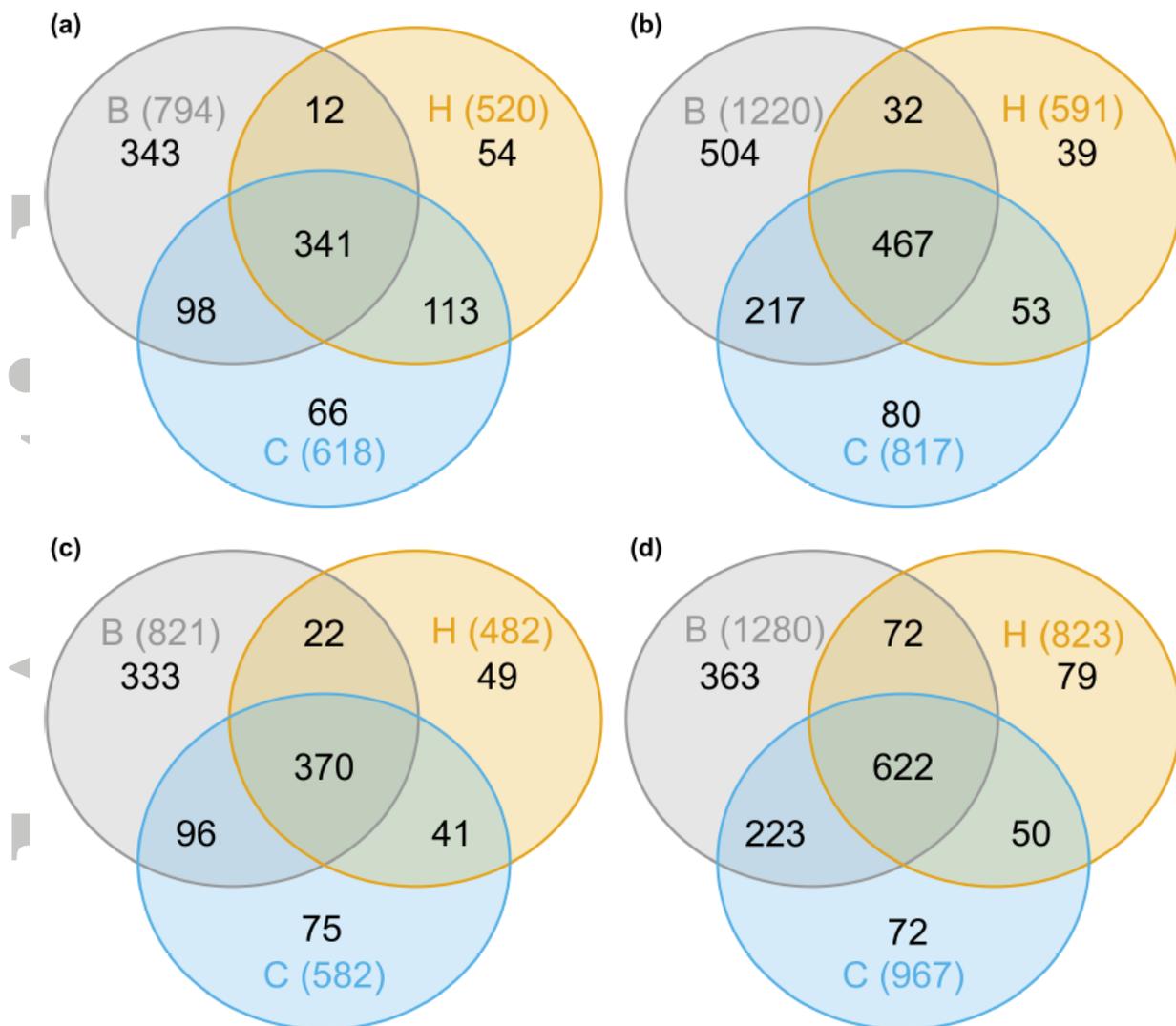


**Figure 4:** Principal Component Analysis (PCA) of lipid abundances normalized to internal standard and fresh weight showing Principal Component (PC) 1 and PC2 of membrane (a, b) or storage lipids (c, d) for the two *A. thaliana* accessions Col-0 (a, c) and N14 (b, d) at different control, priming and triggering conditions. Shown are the scores of log<sub>2</sub> median transformed data as means of three independent experiments, each with five biological replicates.

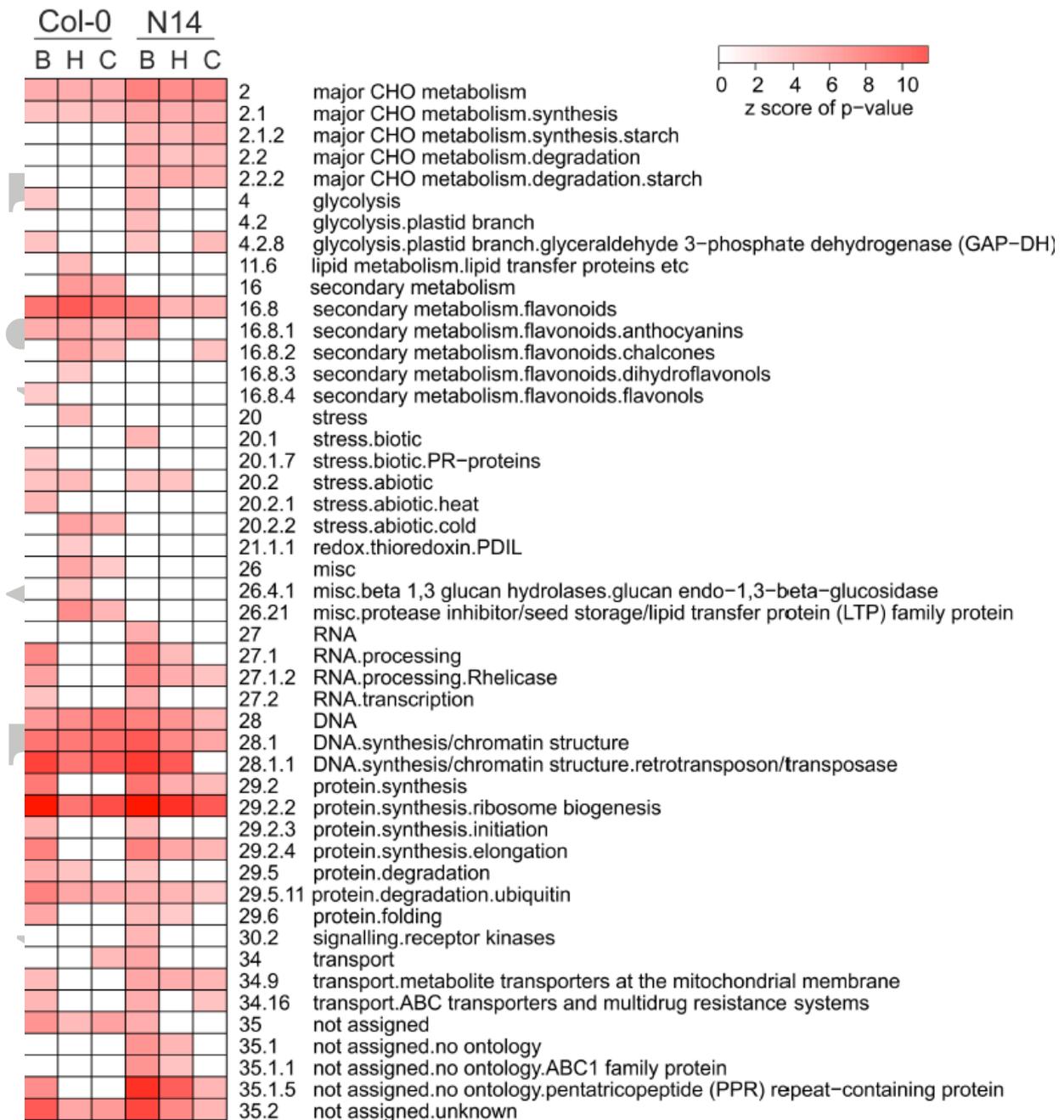


**Figure 5:** Principal Component Analysis (PCA) of metabolite abundances normalized to the internal standard and fresh weight showing Principal Component (PC) 1 and PC2 of 76 overlapping metabolites for the two *A. thaliana* accessions Col-0 (a) and N14 (b) at different control, priming and triggering conditions. Shown are the scores of  $\log_2$  median transformed data as means of three independent experiments, each with five biological replicates.

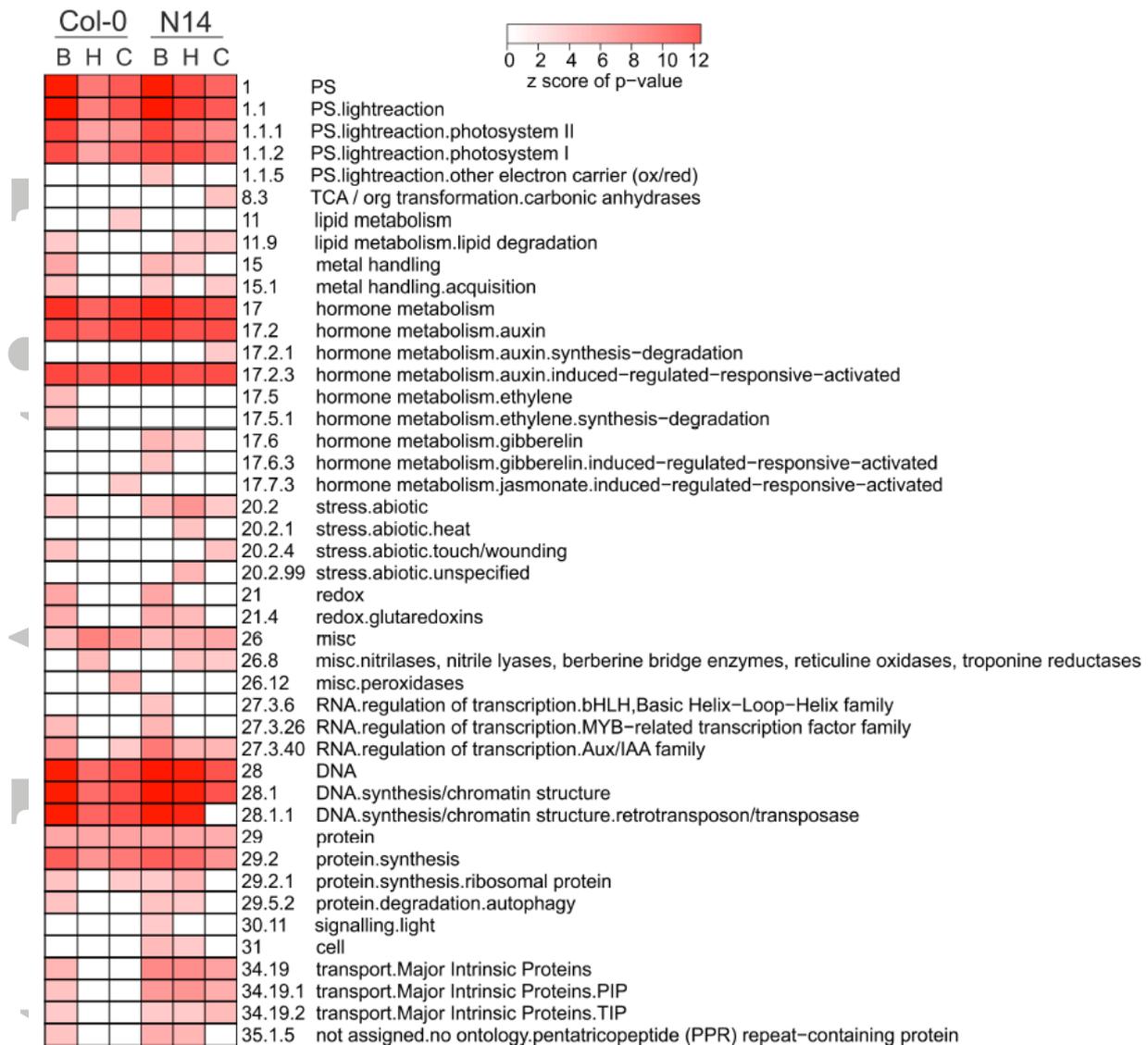
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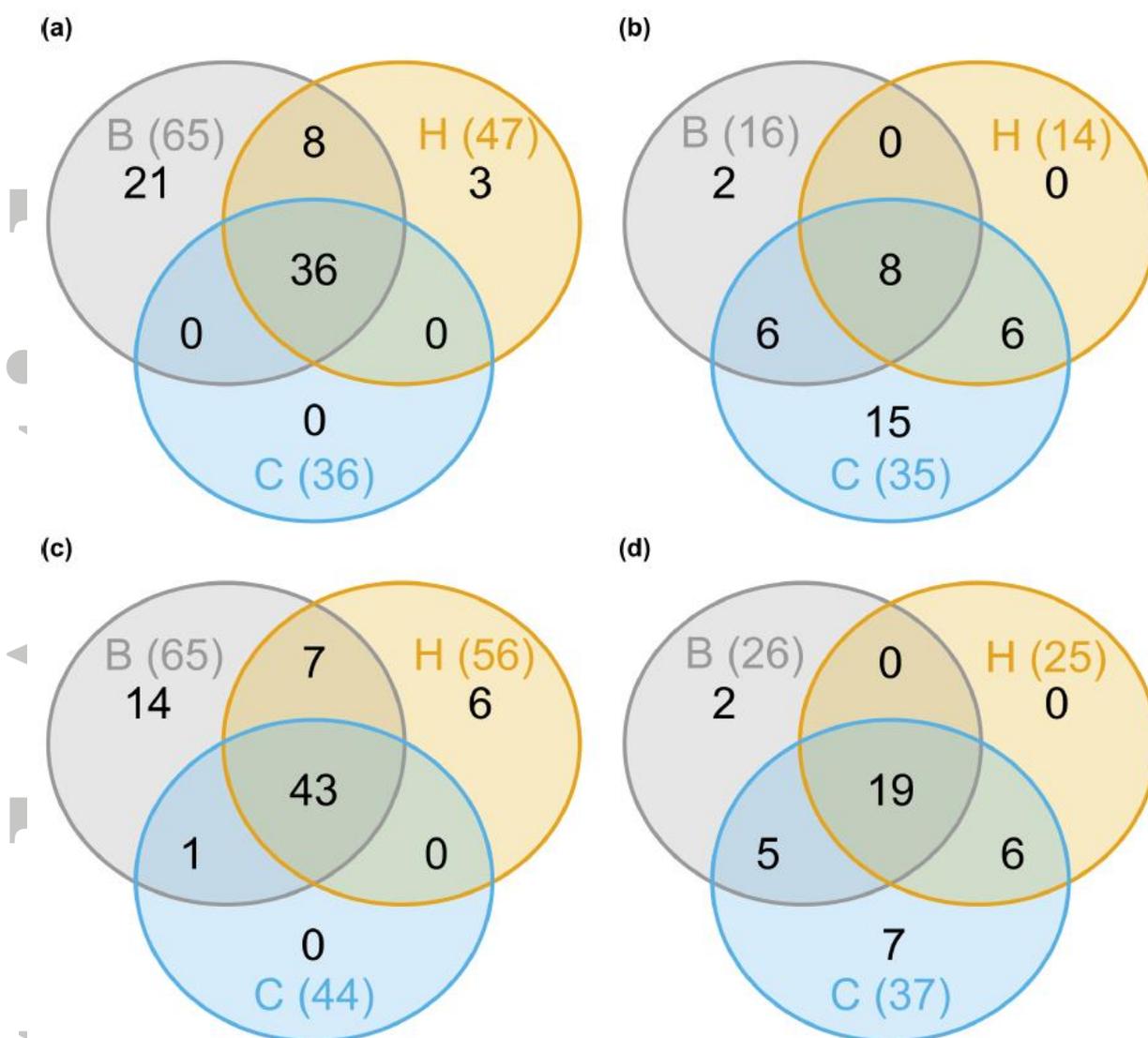
**Figure 6:** Overlap of genes showing significantly increased (a, c) and decreased (b, d) expression in comparison B (grey) (C28P3/C28), H (orange) (C28P3L7T3/C28) and C (blue) (C35P3/C28) for Col-0 (a, b) and N14 (c, d). Numbers in parentheses show the absolute number of genes with significantly changed expression for the respective comparison (compare Table 2).



**Figure 7:** Over-/Underrepresentation analysis of up-regulated genes significantly changed in expression in different functional groups in comparison B (C28P3/C28), H (C28P3L7T3/C28) and C (C35P3/C28) in the accessions Col-0 and N14. Genes were grouped in MapMan bins and overrepresentation of genes showing significant up-regulation of their expression under cold conditions was determined using the Fisher's exact test with multiple testing corrections. Z-scores of transformed p-values ( $< 0.01$ ) are shown. High z-scores indicate a significant enrichment of gene responses in a given functional category.

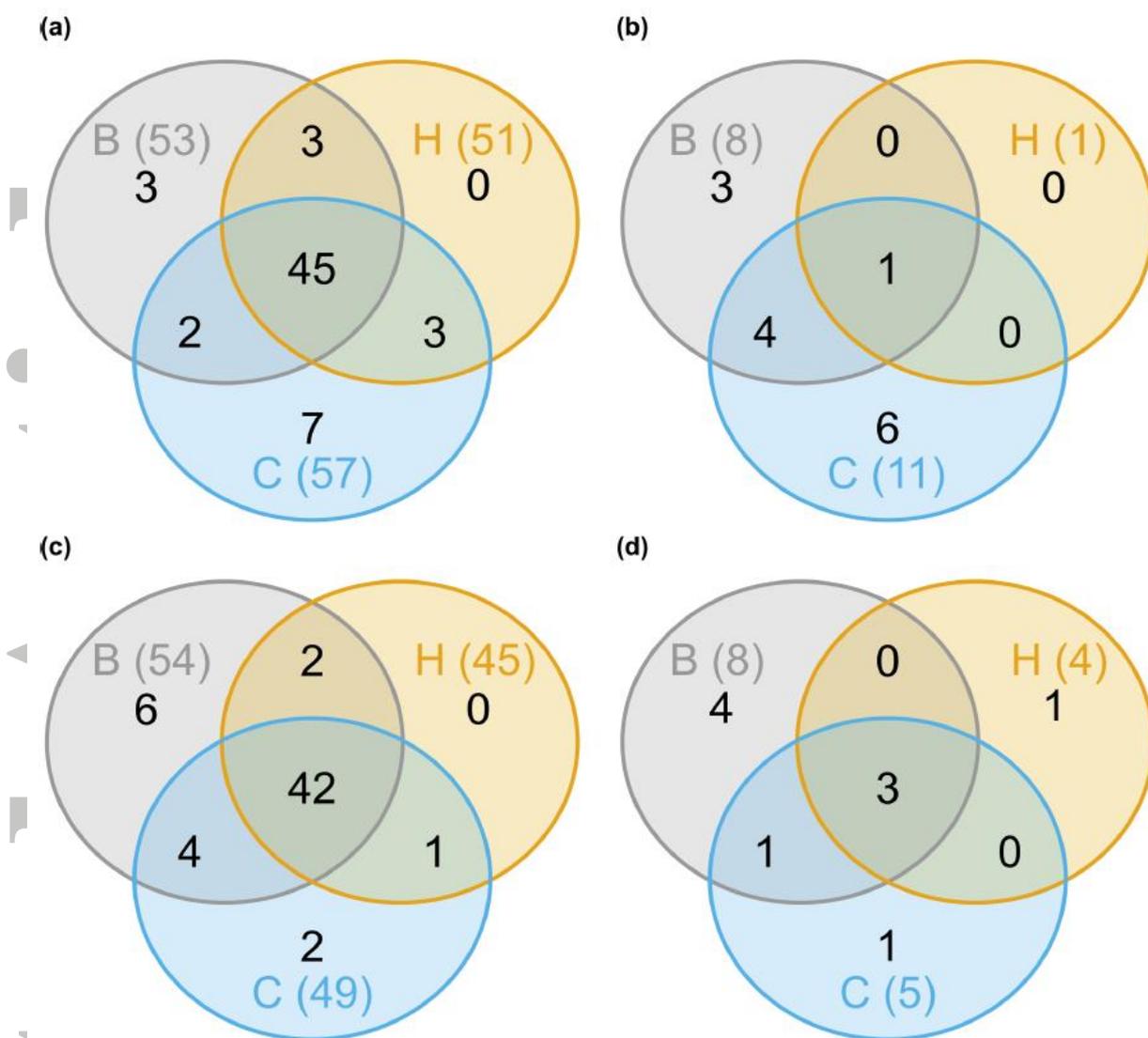


**Figure 8:** Over-/Underrepresentation analysis of down-regulated genes significantly changed in expression in different functional groups in comparison B (C28P3/C28), H (C28P3L7/T3/C28) and C (C35P3/C28) in the accessions Col-0 and N14. Genes were grouped in MapMan bins and overrepresentation of genes showing significant down-regulation of their expression under cold conditions was determined using the Fisher's exact test with multiple testing corrections. Z-scores of transformed p-values ( $< 0.01$ ) are shown. High z-scores indicate a significant enrichment of gene responses in a given functional category.

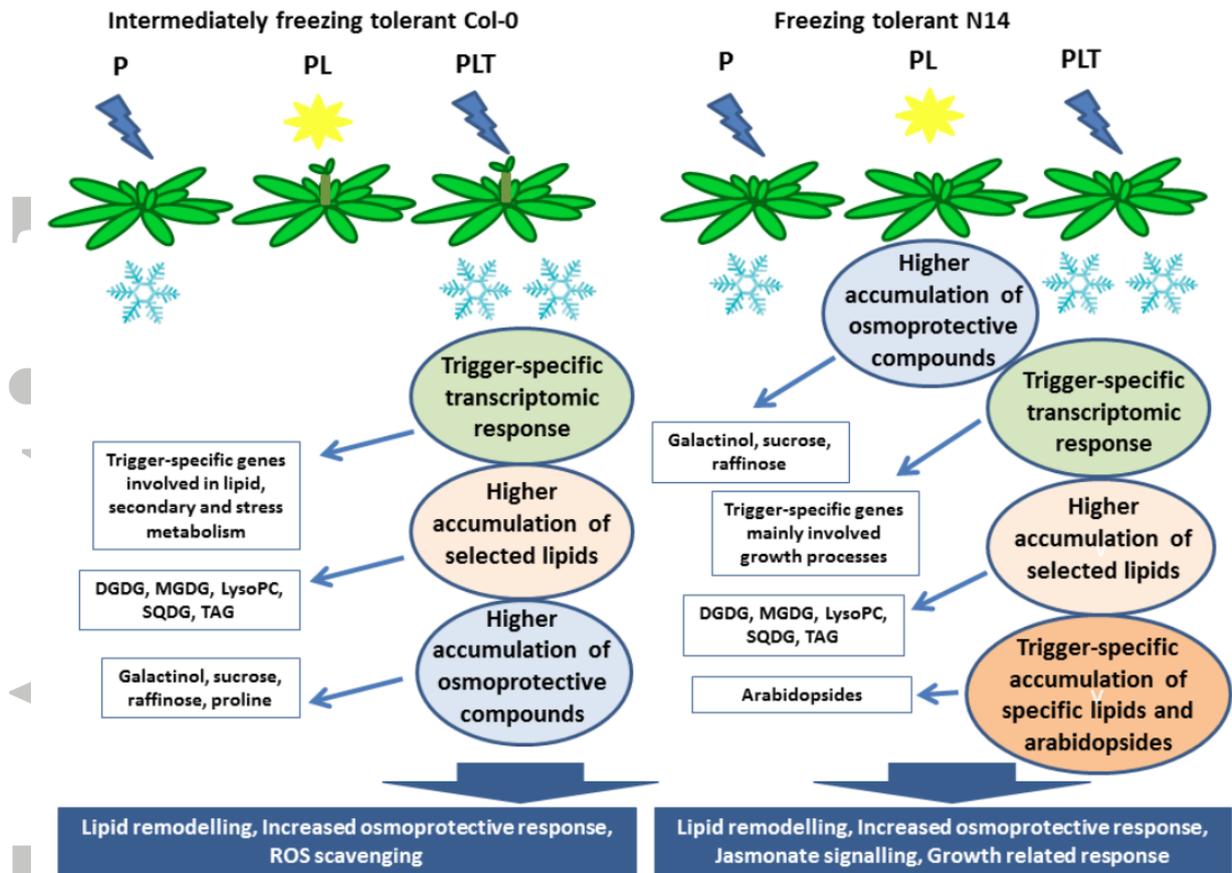


**Figure 9:** Overlap of significantly increased (a, c) and decreased (b, d) lipids of comparison B (grey) (C28P3/C28), H (orange) (C28P3L7T3/C28) and C (blue) (C35P3/C28) for Col-0 (a, b) and N14 (c, d). Numbers in parentheses show the absolute number of significantly changed lipids for this comparison (compare Table 3).

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**Figure 10:** Overlap of significantly increased (a, c) and decreased (b, d) metabolites of comparison B (grey) (C28P3/C28), H (orange) (C28P3L7T3/C28) and C (blue) (C35P3/C28) for Col-0 (a, b) and N14 (c, d). Numbers in parentheses show the absolute number of significantly changed metabolites for this comparison (compare Table 4).



**Figure 11:** Overview of responses associated with memory and triggering in the intermediately freezing tolerant accession Col-0 and the freezing tolerant accession N14.

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## Brief Summary

Cold acclimation results in increased freezing tolerance that is lost upon transfer back to warm conditions. *Arabidopsis thaliana* shows cold memory, leading to higher freezing tolerance during a second cold treatment that is related to specific transcriptomic, lipidomic and metabolomic responses.

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