

**ARAB2010 – UBC Array - Methods**  
**Caterpillars, Aphids, & Mechanical Wounding**

**Plant and Insect Rearing**

*Arabidopsis thaliana* (L.) ecotype Columbia seeds were vernalized in 2% agar and sown into 6 x 5 cm pots containing sterile Metromix 200 soil (Sun Gro Horticulture). Plants were chamber grown at  $22 \pm 1$  °C,  $65 \pm 5$  % relative humidity, and  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity on a 8:16 (L:D) photoperiod. Plants were watered as needed and fertilized every other watering with 21-7-7 Miracle Gro (Scotts Company). The plants were used in experiments 6 weeks after germination; at this time, their rosette diameter exceeded the 2 inch pots but they had not yet started bolting.

The aphids *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer) were maintained as plant virus free clones on pak-choi plants (*Brassica campestris* L. ssp. *chinensis* cv. Black Behi). Eggs of the caterpillar *Spodoptera exigua* Hübner (Noctuidae) were obtained from Benzon Research (Carlisle, PA) and larvae were reared on artificial diet (Bioserv, Frenchtown, NJ, USA). The caterpillar *Pieris rapae* L. (Pieridae) was maintained as a culture in our lab on pak-choi and originated from the Carolina Biological Supply Company (North Carolina). Both caterpillar species were transferred to Col WT plants one day before the experiments to acclimate to the new host. *Arabidopsis thaliana* (L.) ecotype Columbia seeds were vernalized in 2% agar and sown into 6 x 5 cm pots containing sterile Metromix 200 soil (Sun Gro Horticulture). Plants were chamber grown at  $22 \pm 1$  °C,  $65 \pm 5$  % relative humidity, and  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity on a 8:16 (L:D) photoperiod. Plants were watered as needed and fertilized every other watering with 21-7-7 Miracle Gro (Scotts Company).

**Plant Treatments**

Plants were treated with caterpillars and aphids in separate experiments summarized in Table XX. The first sampling of plants occurred several hours after removal of insects so that plant gene expression was not confounded by insect rna or by plant gene expression elicited by the physical movement of insect removal. A summary of the treatments analyzed is given in Table 1.

**Table 1. Treatments analyzed in this study**

| Abbreviation | Insect                       | tissue   | time | # of biological replicates |
|--------------|------------------------------|----------|------|----------------------------|
| Pi-L-6h      | <i>Pieris brassicae</i>      | local    | 6 h  | 3                          |
| Pi-L-24h     | <b>Pieris brassicae</b>      | local    | 24 h | 4                          |
| Pi-S-6h      | <i>Pieris brassicae</i>      | systemic | 6 h  | 4                          |
| Pi-S-24h     | <i>Pieris brassicae</i>      | systemic | 24 h | 4                          |
| Sp-L-6h      | <i>Spodoptera exigua</i>     | local    | 6 h  | 3                          |
| Sp-L-24h     | <i>Spodoptera exigua</i>     | local    | 24 h | 4                          |
| Sp-S-6h      | <i>Spodoptera exigua</i>     | systemic | 6 h  | 4                          |
| Sp-S-24h     | <i>Spodoptera exigua</i>     | systemic | 24 h | 4                          |
| Br-6h        | <i>Brevicoryne brassicae</i> | -        | 6 h  | 4                          |
| Br-24h       | <i>Brevicoryne brassicae</i> | -        | 24 h | 4                          |
| My-6h        | <i>Myzus persicae</i>        | -        | 6 h  | 4                          |
| My-24h       | <i>Myzus persicae</i>        | -        | 24 h | 4                          |
| Wo-L-6h      | Wounding                     | local    | 6 h  | 3                          |
| Wo-L-24h     | Wounding                     | local    | 24 h | 4                          |
| Wo-S-6h      | Wounding                     | systemic | 6 h  | 3                          |
| Wo-S-24h     | Wounding                     | systemic | 24 h | 4                          |

The caterpillar treatment was designed to capture early gene expression events and minimize variation due to leaf age and amount of insect damage. All leaves selected for treatment and harvest were fully-expanded mature leaves. Six to 10 second and third instar *S. exigua* and *P. rapae* caterpillars were allowed to feed for 2-4 hours to generate 6 leaves of similar age per plant with 10-30% leaf area removed. Caterpillars were wrangled as needed with camel hair brushes (size 0) to concentrate their feeding on 6 leaves, and leaves of control plants were jiggled with a camel-hair brush to simulate the leaf movement caused by wrangling. Once sufficient damage was achieved, caterpillars were removed and the plants were returned to the growth chamber. The mechanical wounding treatment was designed to approximate insect damage to tissues by running a sterile pattern wheel across both sides of the midrib of 6 leaves of similar age on each plant, once at the beginning of the caterpillar treatment and again half way through the caterpillar wrangling period. Control plants were jiggled with a camel-hair brush to simulate the leaf movement caused by mechanical wounding. Leaves were harvested for gene expression at 6 and 24 hr after the start of caterpillar damage or wounding. Unwounded leaves were harvested separately from size-matched damaged or wounded leaves. Leaves from 3-4 plants were pooled for each of the four bioreplicates.

Because aphids have effects on plants that are much weaker and slower to develop than those of caterpillars (Mewis et al 2005, 2006) and aphids cannot be readily contained on individual leaves, the design of their treatment was different from that for caterpillars. Twenty sub-adult (final instar) and adult aphids were placed on plants whose rosettes were caged at the soil line by transparent mylar cylinders (5 cm diameter, 9 cm high) with tops of fine mesh gauze (< 0.01 mm mesh wide) to maintain air exchange. Controls were caged plants without aphids, and all plants were returned to the growth chamber. After 1 week of feeding, all cages and aphids were removed and control plants were jiggled with a camel-hair brush to simulate the leaf movement caused by aphid removal. Plants were returned to the growth chamber and whole plants were harvested for gene expression at 6 and 24 hr after aphid removal.

### **RNA isolation**

Total RNA was isolated from leaves using a modified TRIZOL extraction method as follows. Approximately 0.5g of plant material was ground in liquid nitrogen using a mortar and pestle, resuspended in 6 ml TRIZOL reagent (Invitrogen, Carlsbad CA, USA), vortexed and incubated at 65°C for 5 min with regular mixing. Cell debris was pelleted by centrifugation for 30 min at 12,000 g and 4°C and the supernatant was extracted with 3 ml chloroform twice. After centrifugation for 20 min at 12,000 g, the aqueous phase was recovered and RNA was precipitated at room temperature for 5 min with 0.5 volumes of 0.8 M sodium citrate and 0.5 volumes isopropanol. After centrifugation for 30 min at 12,000g, the pellet was washed with 70% ethanol and re-centrifuged. The RNA pellet was air dried for 5 min and resuspended in 200 µl RNase free water. Following a spectrophotometric determination of RNA concentration, the RNA was precipitated with 2.5 volumes of ethanol and a 1/10 volume of 3 M sodium acetate at -20°C overnight, and subsequently pelleted at 20,000 g for 30 min at 4°C. The precipitate was washed with 70% ethanol, re-centrifuged, air dried and resuspended in RNase free water to an approximate concentration of 5µg/µl. Actual concentration was determined spectrophotometrically, and RNA quality of randomly selected samples was determined using a 2100 Bioanalyzer (Agilent Technologies, Mississauga ON, Canada).

### **Microarray design and production**

The *Arabidopsis* Genome Oligo Set Version 1.0 (Operon Biotechnologies, Huntsville, AL, USA) consists of 26090 70mer oligonucleotides (<http://omad.operon.com/arabidopsis/index.php>). As positive controls we included oligos for twelve housekeeping genes (Operon) and as negative controls we included 16 oligonucleotides with no similarity to any *Arabidopsis* gene (4 synthesized human genes and 12 others). Three of these oligos were complementary to the human cRNAs generated as internal standards (spikes, see below). We used a PCR amplified green fluorescent protein (GFP) cDNA (Invitrogen, Carlsbad, CA,

USA) as an orientation marker. Oligonucleotides were resuspended in 384 well flat bottom plates (Nunc, Rochester, NY, USA) to a concentration of 100 mM in 3 x SSC. Oligos were printed on MicroGrid II robots using Microspot 10k pins (Biorobotics, Huntington, UK) depositing approximately 0.1 nl (0.0075 pmole) of each oligo onto EZ rays aminosilane slides (Apogent Discoveries, Hudson, NH, USA). The pitch of the grid used for this library was 0.3 mm. Oligos were UV cross linked at 3000 x 100  $\mu$ J using a UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA). We spotted single spots for the 26090 target; in addition, each subgrid contained six replicate spots of each of the four human negative controls, three spots of equally distributed Operon negative controls, a single spot of each of the twelve house keeping controls, and GFP marker on each corner of the subgrid. The location of each oligo is provided in the platform file to be deposited to the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>).

### **RNA labelling and microarray hybridization**

Total RNA was used for a direct labelling procedure. Eighty  $\mu$ g total RNA was incubated with 0.27  $\mu$ M T<sub>17</sub>VN primer, 0.15 mM dATP, dCTP, and dGTP, 0.05 mM dTTP (Invitrogen), 0.025 mM Cyanidin3- or Cyanidin5-conjugated dUTP (Amersham, Piscataway, NJ, USA), 40 U RNaseInh (Promega, San Luis Obispo CA, USA), and 400 U SuperscriptII (Invitrogen) in 10 mM DTT and 1 x first strand buffer in a total volume of 40  $\mu$ l. In addition, 0.3 fmole human cRNAs complementary to the human negative control oligonucleotides were used in labelling reactions (HsD17B1, KRT1, and MB). Prior to addition of enzymes the solution was heated to 65°C for 5 min and for primer annealing cooled to 42°C. Following an incubation at 42°C for 2.5 h, the RNA was degraded with 8  $\mu$ l 1 M sodium hydroxide for 15 min at 65°C, neutralized with 8  $\mu$ l 1 M hydrochloric acid and buffered with 4  $\mu$ l 1M Tris-pH 7.5. Subsequently, the labelled cDNA was purified using a PCR purification kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). DNA was eluted in 100  $\mu$ l 10 mM Tris, pH 8.5, the two labeling reactions were combined, and 1  $\mu$ l Cyanidin5-labelled GFP was added. Following an ethanol/sodium acetate precipitation (Sambrook and Russel, 2001) the air-dried cDNA pellet was resuspended in 3  $\mu$ l water, denatured at 95°C for 3 min, added to 50  $\mu$ l pre-warmed array hybridization buffer #1 (Ambion, Austin, TX, USA), and kept at 65°C until use. We pre-hybridized microarray slides for 45 min at 48°C in 5 x SSC, 0.1 % SDS, 0.2 % BSA. Slides were washed twice with water for 1 min, dipped 5 times in isopropanol, and spun dry in Falcon tubes at 100 g for 3 min. The hybridization solution was applied to the microarray slides and covered with untreated glass cover slips (Fisher Scientific, Nepean, ON, Canada). Arrays were incubated over night in CMT hybridization chambers (Corning, Corning, NY, USA) submerged in a water bath at 42°C with moderate vertical shaking. Hybridization chambers were disassembled and slides were washed for 15 min at 42°C in 2 x SSC, 0.5 % SDS, and for 2 times 15 min in 0.5 x SSC, 0.5 % SDS. Subsequently, arrays were dipped five times in 0.1 x SSC and spun dry as described above. Microarrays were scanned with a ScanArray Express (Perkin Elmer, Woodbridge, ON, Canada) scanner with laser power set to 95% and photo-multiplier-tube set at 54 to 64.

### **Hybridization design and raw data organization**

For each treatment, three to four replicate arrays were hybridized for a total of 64 arrays. RNA from the controls harvested with each treatment was pooled within time points to obtain sufficient control RNA. Labelled cDNA derived from the pooled control RNA was co-hybridized with labelled cDNA derived from RNA isolated from independent bioreplicates receiving insects or mechanical wounding. This results in four biological replicates for each treatment. To account for possible dye biases, within each treatment group the dye labeling was swapped between treatment and control samples among bioreplicates. Hybridization orders were randomized to avoid biases due to the hybridization time.

### **Data analysis**

We identified and quantified spots using ImaGene software (BioDiscovery, Marina Del Rey, CA, USA). Grids were manually placed and spot finding was performed using the 'Auto adjust' spot function repeated three times. Spot finding was subsequently verified by visual inspection and manually adjusted

when necessary. Weak spots (average signal intensity less than three standard deviations over local background intensity) were automatically flagged (flag 2), and poor spots were manually flagged (flag 1); good spots were flagged as 0. Spots with a flag 1 were not used in further data analyses. For all analyses, the median pixel intensities for each spot were used. The raw data files will be deposited into the GEO database. Further analyses were performed with gene specific elements only using customized scripts for R and Bioconductor (The R Development Core Team, [www.r-project.org](http://www.r-project.org)). For background correction, we defined the mean of the lowest 10% of spot intensities from a particular subgrid as the background for that subgrid. This mean was subtracted from each spot in the subgrid, and all spots with original signal intensities below a threshold comprised of the background plus three standard deviations of the background in each subgrid were omitted from further analysis. On average 19% of all spots (ranging from 12% to 27%) were thus excluded from further analyses as non-detectable. Background corrected signal intensities were used for Loess normalization (Yang *et al.*, 2002), to generate normalized  $\log_2$  expression ratios comparing each treatment with the corresponding control sample. For each element, we first used the data from the four replicate arrays for each sample to perform a paired Student's t-test using the Welch approximation to the degrees of freedom and to calculate mean expression ratios for each treatment. Subsequently, an analysis of variance (ANOVA) using data from all experimental samples was performed for each element. In order to assess the type I error rate, we calculated q-values estimating the false discovery rate based on the parametric p-values (Storey and Tibshirani, 2003). For initial cluster analyses of the expression data, genes were first filtered based on the p(t-test) value and only genes with  $p < 0.05$  and a fold-change of more than three between treatment and control in at least one sample were used. We then used the normalized expression ratios from all treatments to perform an analysis of variance (ANOVA) and again estimated the false discovery rate based on the distribution of parametric p-values (Figure SXX). The means of the normalized expression ratios were subjected to a hierarchical cluster analysis with average linkage using Genesis v1.2 (Institute for Biomedical Engineering, Graz University of Technology, Graz, Austria).

### **Metabolite Extraction**

All plant samples are received ground and freeze-dried in 1 mL centrifuge tubes. Samples masses are approximately 15 mg—exact masses are recorded in data spreadsheet. To each centrifuge tube is added 1 mL 50/50 methanol/water to which has been added 13.3 mg/mL of each of the internal standards. The tray of sample tubes is wrapped in foil and stored at 9°C for 24 hrs, at which point the supernatant is transferred to autosampler vials for LCMS analysis.

### **Chromatographic Separation**

All samples were run on a Shimadzu (Kyoto, Japan) SCL-10ADvp HPLC system with a Thermo Electron (Bellefonte, PA) Betabasic C18 (150 x 1 mm; 5 mm particle size) reverse phase column connected directly to the ion source inlet. Chromatographic separation was achieved using elution solvents A = water with 0.15% v/v formic acid, B = methanol. Initial conditions were 1% B and the solvent gradient began at 0 min and ramped to 100% B over 37 min. It was then held at 100% B for 6 min. The flow rate was approximately 100 mL/min and each injection consisted of 10 mL plant extract.

### **Mass Spectrometric Analysis**

The HPLC was coupled to a Micromass (Manchester, UK) Quattro II mass spectrometer. The instrument was equipped with an electrospray ionization probe and was operated in both the negative and positive modes. The mass spectrometer was run with an alternating cone voltage experiment, such that two spectra were acquired at every time point—one at low cone voltage (20 V) and one at high cone voltage (75 V). In this way, in source collision induced dissociation (CID) was achieved resulting in both molecular ions and the corresponding fragment ions in separate spectra. For negative mode experiments, the scan range was 100-1000 m/z, whereas for positive mode experiments, the scan range was 100-1500 m/z. The source block temperature was 100°C. The source capillary voltage was -2.5 kV.

**Data Analysis**

All chromatograms were processed in the MassLynx. Using the MassLynx method editor, selected single ion chromatograms (SIC) were integrated and that peak area information is tabulated in the Excel spreadsheet. Particular attention was paid to the glucosinolates and polyphenolics. The raw peak area was then adjusted for sample size and to the nearest internal standard. The adjusted data was then normalized to 100. Both low and high cone voltage spectra are included for each of the compound integrated. When helpful, the low cone voltage spectra with the background subtracted are also included as well as expanded spectra showing the molecular ion for the glucosinolates.