Global Changes in Gene Expression during *Alstroemeria* Petal Senescence

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Abstract

Global patterns of gene expression were examined in *Alstroemeria* flowers after harvesting at the closed bud stage. A number of cDNA libraries were made and limited sequencing allowed some functional analysis to be performed. The design and statistical analysis of microarray experiments from these clones is described and showed good correlation between replicates and good signal detection limits. 77% of the genes represented on the array were above a 10% threshold of detection in all replicates in stages 0, 2 or 5. K-means clustering was used and several distinct patterns of expression were identified.

INTRODUCTION

Post harvest senescence of *Alstroemeria* flowers has been studied at the physiological level (Wagstaff et al., 2001; Chanasut et al., 2003), ultrastructural level (Wagstaff et al., 2003) and some aspects of protein degradation and lipid peroxidation have been investigated at the molecular level (Wagstaff et al., 2002; Leverentz et al., 2002). The advent of new technologies has enabled the molecular control of plant development to be examined in more detail and with fewer preconceptions of the specific genes involved. We have now applied microarray analysis to post harvest senescence using a customised microarray to facilitate the identification of changes in global gene expression in *Alstroemeria*. The data obtained will provide new insights into the identity of a variety of genes whose expression pattern changes during senescence but, by ontological analysis, specific pathways can also be identified.

MATERIALS AND METHODS

Floral Material and RNA Purification

Alstroemeria (var. Rebecca) flowers were used throughout the experiments and were obtained from Oak Tree Nursery, Egham, UK. Inflorescences were harvested at stage 0 before transporting back to the laboratory dry (c. 20 min). Individual cymes were isolated and maintained in dH_2O and the top two petals were removed from a number of flowers at each stage and immediately ground in liquid nitrogen in preparation for RNA extraction. RNA was isolated from *Alstroemeria* petals at eight stages of flower opening and senescence. The stages were defined by morphological characteristics (Table 1, reproduced from Wagstaff et al., 2003). RNA extraction used Tri-reagent (Sigma, UK) according to the manufacturers protocol, but with an additional step of phenol:chloroform:isoamylalcohol (25:24:1) separation of nucleic acids in the aqueous phase and subsequent ethanol precipitation. Messenger RNA was purified from total RNA using Dynabeads (Dynal, UK) according to the manufacturer's instructions.

cDNA Library and Microarray Construction

cDNA libraries were made using the Stratagene λzap gigapack gold kit from 5μg mRNA starting material. Three libraries were made: stages 0 and 1 combined; stages 2-4 combined and stages 5-7 combined. A mass excision was performed from each library and clones were grown overnight on selective plates. Approximately 1000 clones were isolated from each library and stored in the form of glycerol stocks at -80°C. The glycerol

stocks was used to inoculate a 1.5ml overnight LB culture and plasmid DNA was purified by miniprep (Whatman) and inserts were amplified by PCR. PCR cleanup was performed using Whatman kits and each product was then quantified by gel analysis. A 96-well plate of PCR products was sequenced from each library. All (6513) PCR products were diluted by 50% with DMSO and printed in duplicate onto Corning glass slides in the form of a microarray. Quality control was performed on one slide from each print run and landmarks and known genes were included on the microarray.

Probes for microarray analysis were made using Cyscript direct labelling kits (Amersham) and experiments were performed in triplicate with reciprocal cy3/cy5 labelling. Hybridisation was performed overnight at 42°C on an automatic hybridisation station (GeneTac) and slides were scanned using GeneTac GLS software. Spot intensities were quantified using Imagene (BioDiscovery) and subsequent normalisation and analysis was performed with GeneSpring software (Silicon Genetics).

Analysis by Venn Diagram

In order to generate a diagram showing genes consistently expressed above a certain level the following statistical analysis was performed. The background value was subtracted from the signal value for each spot. The mean value for each pair of spots corresponding to a single clone on each slide was determined. The mean, signal standard deviation (precision) and co-efficient of variation across the six technical replicates (three slides) for each stage was calculated. The mean signal per spot per slide was then determined and all clones that did not have a mean signal value greater than 1/10 of this value in any of the stages examined were presumed to be absent. All clones that had a co-efficient of variation within triplicates greater than 100% were also removed from the dataset so the resultant set of clone identities represents genes that show a detectable and statistically reliable signal in all spots throughout the experiment.

K-means Clustering

K-means clustering was performed using GeneSpring using a number of statistical correlations which all gave similar results. Clusters are formed by an iterative process of grouping genes with expression patterns that are more similar to each other than to any other clustered groups. The result of k-means clustering based on a smoothed correlation using eight clusters is shown in this paper.

RESULTS

Three cDNA libraries were constructed from different developmental stages of *Alstroemeria* flower opening and senescence. Only the top petals of the first flower on each cyme were used to maximise tissue specificity of the clones isolated. The first library was from opening buds (stages 0 and 1), the second from fully open flowers (stages 2, 3 and 4) and the third from senescing flowers (stages 5, 6 and 7) (Fig. 1). At least 1000 clones were isolated from each library and approximately 1/10 of these were sequenced to determine insert size, numbers of doublets and the level of redundancy within the libraries. The only sequence identity that had a high level of duplication related to a gene encoding metallothionien-like proteins.

The sequences were grouped according to function for each library (Fig. 2). The analysis shows that signalling, development and metabolism-related genes are most prevalent in the younger libraries, whereas metal binding genes are more common in the library made from petals between S2 and S7. Transcription factors are present at a constant level in all three libraries.

The cDNA inserts were amplified from each clone by PCR and purified before spotting in duplicate on glass slides. Microarray experiments were performed using stages 0, 2, 3 and 5 as probes labelled with cy3 and cy5 fluorescent dyes. Three replicates of each stage were analysed using GeneSpring software and normalisation was performed between replicate spots within and between slides. The variation between replicates was determined. Of the 6513 genes represented on the arrays, for S0 over 41% failed to give

good agreement (less than three-fold variation in normalised values) between replicate slides suggesting either poor reliability of the technique or non-expression at this stage. The figures from similar analyses of stages 2, 3 and 5 were 17%, 22% and 18% respectively. The high values for the comparison in S0 compared to the other stages suggests that many genes showed little or no expression at this stage of development. This hypothesis was supported by analysis of changes in expression levels during development (see below). Analysis of all genes on the array showed a range of expression patterns (Fig. 3). The sets of genes showing a significant level ($p<0.05$) of expression at stages 0, 2 and 5 across all six replicates were compared by a Venn diagram (Fig. 4). This shows that 77% of the genes in the experiment gave a detectable signal (greater than 10% of the average signal across all replicates) with a low co-efficient of variation (less than 100%). Of these genes, the largest proportion (3888 genes or 78%) were common to all stages, 164 were unique to one stage only and 439 were common to two out of the three stages.

Genes were separated into eight different expression patterns by K-means clustering analysis based on a smoothed correlation (Fig. 5). Analysis of the genes in these classes shows that 10.5% (class a+b) are continuously down-regulated between S0 and S5; 25.8% (c+d) are continuously up-regulated over the same period; 42.3% (e+f) are up-regulated between S0 and S2 only; 12.0% (class g) peak expression at S2 and 9.4% (class h) show no differential expression during the course of the experiment.

DISCUSSION

Microarray analysis is becoming the technology of choice for many researchers interested in changes in global gene expression patterns in organisms exposed to different environmental conditions, or at different stages of development. It has proven particularly useful in instances where a number of pathways are expected to show altered regulation between conditions and a gene-by-gene approach is neither practical, nor representative of the dynamic state of the organism under study. In this paper we present data for post-harvest analysis of *Alstroemeria* using our own printed cDNA microarrays. The arrays were constructed from several developmental libraries in order to ensure that a wide range of genes that may be specific to different stages were represented on the arrays.

A limited functional analysis was performed on approximately 10% of genes spotted from each library. The analysis showed similar functional groups as other large-scale sequencing projects on ageing leaf tissue (Buchanan-Wollaston et al., 2003; Gibbings et al., 2003) and demonstrated that processes within the plant are represented by different proportions of genes as senescence progresses. This comparison also indicates that the processes involved in petal senescence are very similar to those found in monocotyledonous and dicotyledonous leaf senescence. The trend is by no means towards overall loss of gene activity. Gene representative of metabolic processes tend to reduce as the organs age. Genes encoding proteins that are involved in degradative processes increase in representation (copy number) within the older libraries, correlating with known examples where transcription and translation occur until the late stages of the senescence process (Buchanan-Wollaston et al., 1994; Thomas et al., 2003). Likewise, the abundance of metallothionein-like genes has been identified before in senescing plant tissues and a type 3 metallothionein was found to be the most abundant transcript found in a recent SAGE analysis of rice leaves (Gibbings et al., 2003).

Analysis of the initial array data obtained from these primary experiments with post-harvest developmental tissue indicated that spot intensity was above a lower threshold of detection in 77% of the genes represented in the majority of replicates. This correlates well to a similar analysis of signal detection across replicates in a spotted *Arabidopsis* array where 80% of spots on the arrays generated detectable fluorescent signals (Ruan et al., 1998). However, compared to leaf and root tissues, *Arabidopsis* flowers showed the least differential expression with only 4% of transcripts showing a two to threefold change in expression between buds and open flowers. In the present *Alstroemeria* experiments the youngest stage examined had the lowest levels of expression, and also the highest number of genes uniquely producing a signal at this stage

only. This potentially indicates that some of the post-harvest processes are occurring at this stage and are complete by the time the flower is fully open, a fact supported by recent ultrastructural evidence in this species (Wagstaff et al., 2003).

Several distinct patterns of gene expression were identified by k-means clustering from our data with more genes showing upregulation between stages 0 and 5 than down regulation over the same period. Although clustering has its limitations (Tilstone, 2003) it is used by many workers to group data together in a visually acceptable form to aid interpretation in a way that long lists of genes cannot (Eisen et al., 1998). Where clustering techniques such as k-means and self-organising map (SOM) are compared, very little variation is found in the clusters obtained and usually only one method is presented (Zhu et al., 2001). Eisen et al. (1998) and Zhu et al. (2001) both obtained evidence that co-regulating genes tend to cluster together. This information can be useful in assigning putative function to the many genes of unknown function that are inevitably identified in these types of post-genomic analyses. As more sequence information in *Alstroemeria* becomes available we anticipate being able to make more informed judgements on how senescence is regulated in petals of these flowers after harvest.

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Tables

Table 1. Morphological stages of *Alstroemeria* development and senescence.

Stage	Day relative to flower opening	Description of floral features
1	-2	Outer sepals pigmented. Tips of sepals loosening.
2	0	Sepals reflexed. No anthesis.
3	+2	Upper three anthers bent upwards and anthesed.
4	+4	Lower three anthers bent upwards and anthesed.
5	+6	Separation and reflexing of stigmatic lobes.
6	+8	Discolouration of petals. Translucence around margin of sepals. Reproductive organs lying on lower petal.
7	+10	Abscission of petals and sepals when lightly tapped.

Figures

Fig. 1. Stages of development in *Alstroemeria* flowers cv. Rebecca.

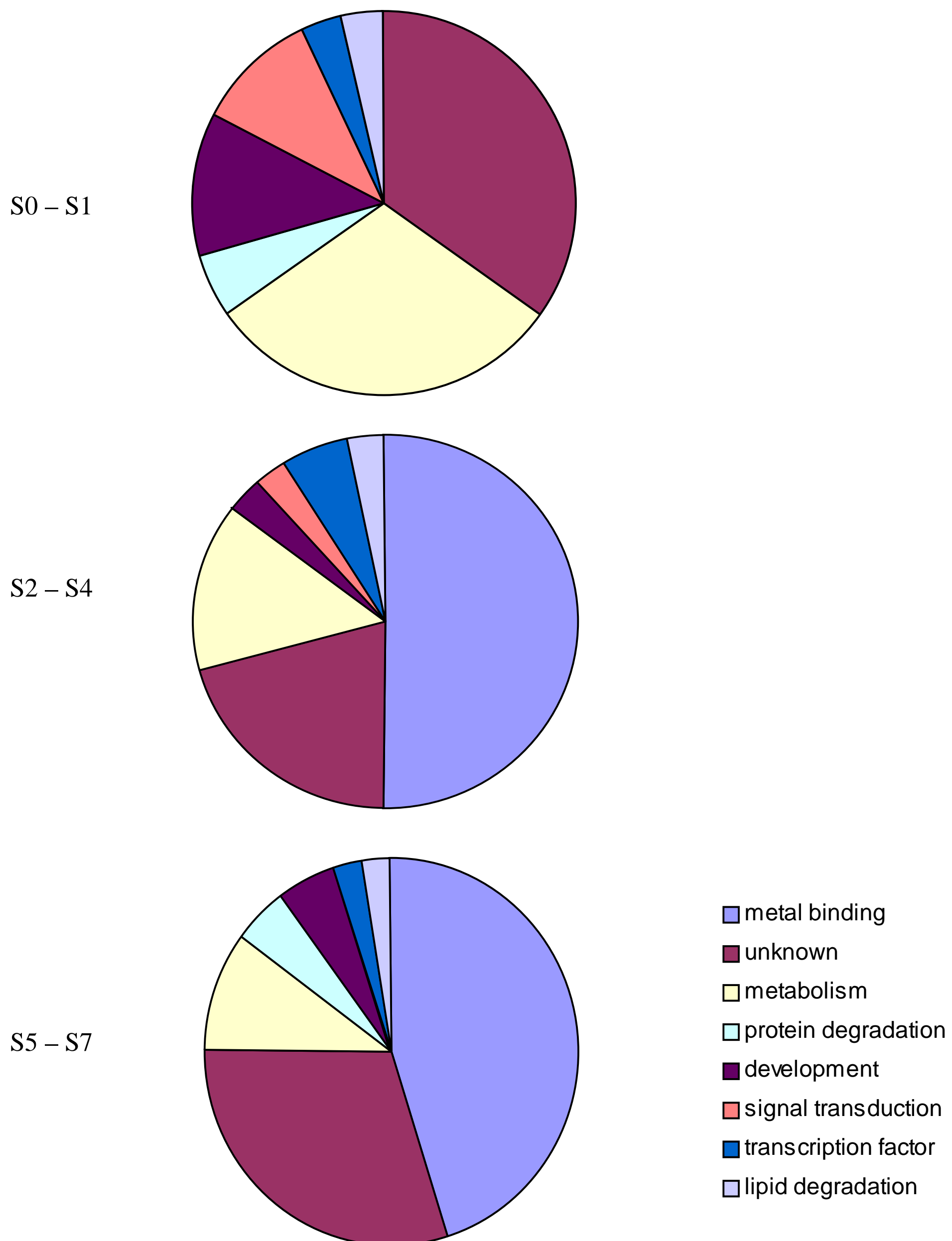

Fig. 2. Functional analysis of sequences obtained from developmental libraries of *Alstroemeria* petals. Approx 1/10 clones selected for arraying were sequenced.

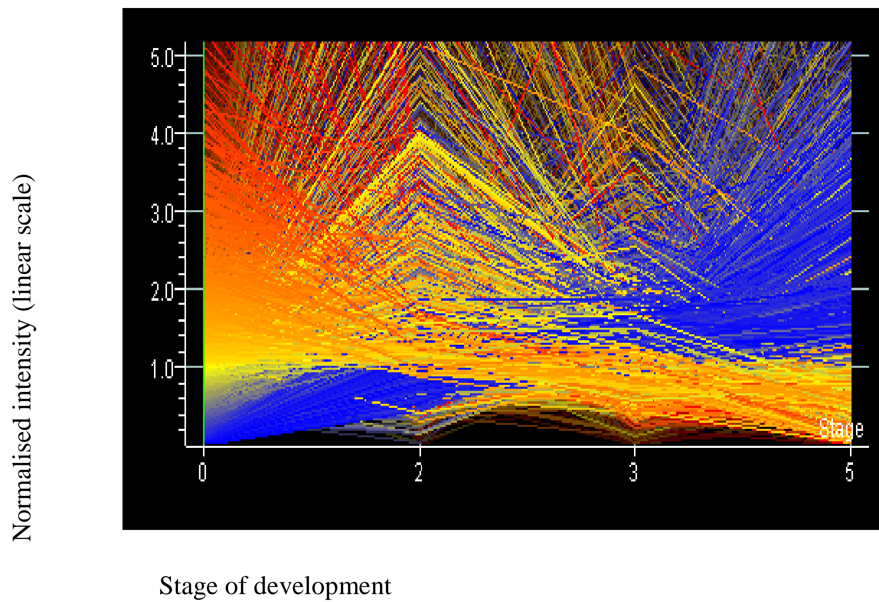

Fig. 3. Gene expression of 6513 genes between stage 0 and stage 5 in a microarray experiment. Genes are colour coded such that lines in red indicate down-regulated genes, blue indicates up-regulation. Bright lines show high levels of confidence (low p values) and dull lines represent those genes with lower confidence levels.

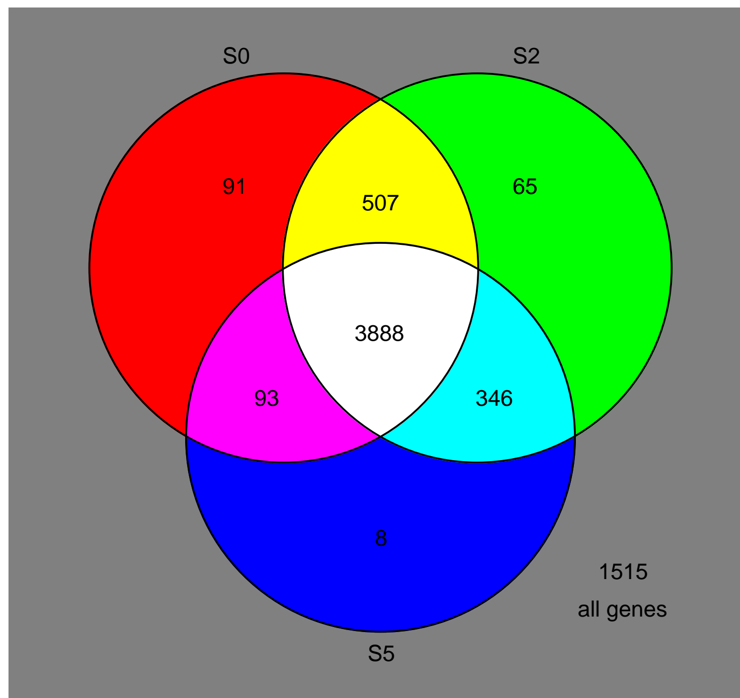

Fig. 4. Venn Diagram depicting genes showing a significant level of expression at stages 0, 2 and 5 across all six replicates. Genes within the circles have a detectable signal that was greater than 10% of the average signal across all replicates at that stage with a co-efficient of variation less than 100%.

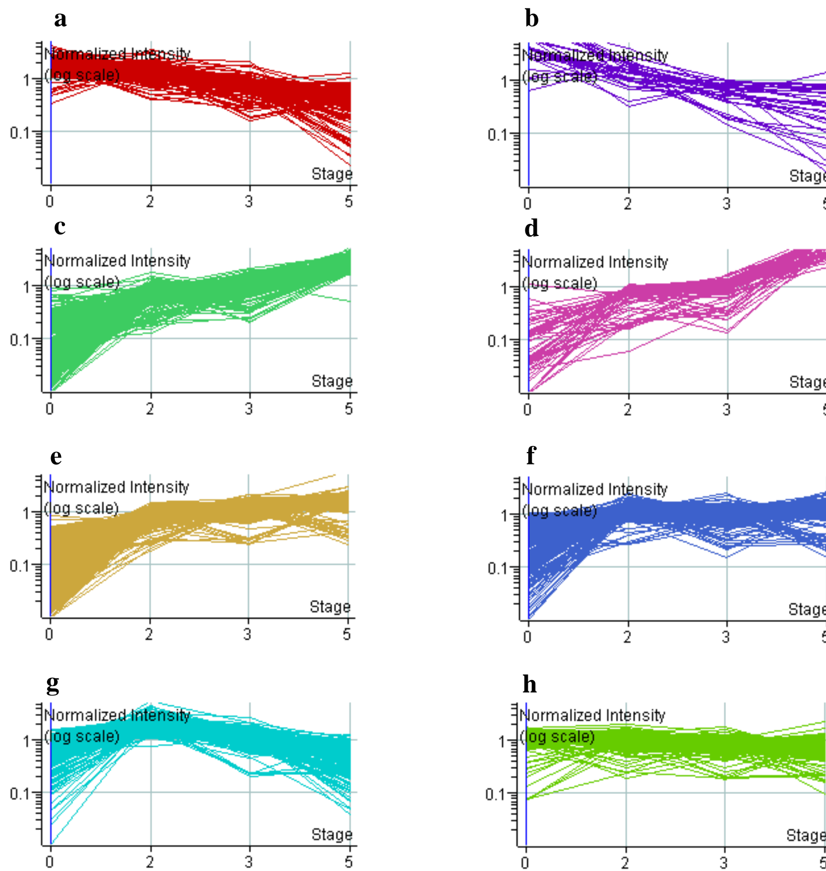

Fig. 5. K-means clustering of genes with a smoothed correlation. Classes a and b are continuously down-regulated between S0 and S5; classes c and d are continuously up-regulated over the same period; classes e and f are up-regulated between S0 and S2 only; class g has a peak of expression at S2 and class h shows no differential expression during the course of the experiment.