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Flower senescence in composite flowers, can understanding how dahlia florets senesce help to increase dahlia vase life?

M. Casey¹, K. Tansey², R. Andrews², A. Marchbank³ H. J. Rogers³, A.D Stead¹

¹ Biological Sciences, Royal Holloway, University of London, Egham, Surrey, TW20 0EX, UK;

² College of Biomedical & Life Sciences, Cardiff University, Sir Geraint Evans Building, Academic Avenue, Cardiff, CF14 4XN

³ School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AT, UK

Abstract

The dahlia is popular as an ornamental garden plant in the UK, however its value as a cut flower has been undermined by its short vase life. The vase life of dahlias is often no more than 5 days, whereas 10-14 days are required by the cut flower industry due to the supply chain involved in transporting cut flowers from growers to retailers, sufficient time in store, and still guaranteeing 5 days vase life to a consumer. The current study has considered ethylene sensitivity and how phytohormones interact with one another during the senescence process. In conjunction with these traditional methods RNA sequencing and de-novo assembly of the dahlia transcriptome in the cultivar 'Sylvia' has been carried out resulting in an assembly of over 20 000 genes, many of which change in expression during floret senescence.

Keywords: flower senescence, ethylene, cytokinins, transcriptome

INTRODUCTION

Dahlias are common garden plants in temperate and tropical regions of the world but are on the fringe of the cut flower market due to their limited vase-life. The cut flower industry involves a supply chain that encompasses growing, harvesting, transportation and storage, retail, and finally a consumer stage. Vase life refers to the consumer stage, but the flowers must last long enough after harvest to be transported from the grower to the packer, spend 2-3 days in the store, and still have a reasonable vase life after this - usually a minimum of 5 days. Ideally therefore flowers need to last for 10-14 days overall. Previous work indicates that the dahlia flower may reach a vase life of up to 10 days (Dole *et al.*, 2009) however, the commercial practicalities of this have yet to be examined.

Ethylene is ubiquitous in plant senescence processes but its role in dahlia flower senescence remains unresolved. Woltering and van Doorn (1988) concluded that dahlias were slightly sensitive to ethylene. Moreover, Dole *et al.* (2009) found that 'Karma Thalia' dahlias were unaffected by an ethylene exposure of 16 h at 1 $\mu\text{L L}^{-1}$. However, Kato *et al.*, (2002) found that *Campanula medium* flowers were unaffected after exposure to ethylene at 10 $\mu\text{L L}^{-1}$ for 16 h, but all flowers wilted after exposure to ethylene at 2 $\mu\text{L L}^{-1}$ for 48 h. Therefore, longer exposure to ethylene may affect dahlias as well and the response may be related to the stage of development when exposed to ethylene. Dole *et al.* (2009) also reported that cut dahlia 'Karma Thalia' stems were unaffected by treatments with STS (silver thiosulphate) or 1-methylcyclopropene (1-MCP) also suggesting these dahlias are insensitive to ethylene. However, other reports show contrasting results. Shimizu-Yumoto and Ichimura (2013) found that cut dahlia flowers ('Kokucho') continuously placed in 1 or 10 $\mu\text{L L}^{-1}$ 2-chloroethylphosphonic acid (CEPA) solution, which generates ethylene, wilted earlier than those treated with distilled water or citric acid. Furthermore, a pulse treatment with 1-MCP extended the vase life. Treatment with cytokinins seems to be effective in delaying dahlia senescence (Shimizu-Yumoto and Ichimura, 2013). A pulse treatment with BA (6-benzylaminopurine) extended floret vase life, and BA was more effective than 1-MCP when the flowers were exposed to ethylene via CEPA treatment (Shimizu-

Yumoto and Ichimura, 2013). BA spray treatment also extended vase life of cut dahlia ('Kokucho', 'Kamakura', and 'Michan'; Shimizu-Yumoto and Ichimura, 2013). Thus, dahlia flower senescence may be only partially regulated by ethylene, and BA may be more effective in delaying the senescence of cut dahlia flowers than ethylene action inhibitors such as STS. Most evidence points to dahlias being ethylene-insensitive, with some slightly sensitive cultivars. Further work is clearly needed to compare ethylene sensitivity amongst dahlia cultivars to establish the range of sensitivities.

Data on dahlia's transcriptome is very limited. Genes implicated in flower pigmentation, such as anthocyanin pathway enzymes, are among the only ones to have been cloned (Suzuki *et al.*, 2002; Ohno *et al.*, 2011, 2013) and characterised (Yamaguchi *et al.*, 1999; Ogata *et al.*, 2001). Whole reference transcriptomes have been generated for the dahlia but focused on a mixture of different organs (Hodgins *et al.*, 2014) or on early flower development (Lehnert & Walbot, 2014). De-novo assembly transcriptomics is a relatively cheap way of gathering large amounts of novel information about gene expression. The Trinity *de novo* assembly method has been widely used since its inception to assemble transcriptomes of non-model species without a reference genome, including in plants (Grabherr *et al.*, 2011; Ward *et al.*, 2012), and transcriptomic changes in flowers (Broderick *et al.*, 2014). In this study RNA-sequencing focused solely on gathering data from dahlia florets of different stages of development in order to gain insight into transcriptomic changes at different stages of a dahlia flower's lifespan. This study also assessed the sensitivity of the popular UK dahlia 'Karma Prospero' to cytokinins and ethylene.

MATERIALS AND METHODS

Materials

Dahlia tubers were obtained from Rose Cottage Farm (Essex, UK). The cultivars 'Karma Prospero' and 'Sylvia' were grown outside at Royal Holloway University of London, Egham, UK. Tubers were planted into 20 cm wide pots filled with peat-based compost in March and allowed to grow in a polytunnel. In the last week of May the plants were removed from their pots and transplanted outside. For postharvest treatments, flowers were harvested at stage III (Fig. 1) as it is claimed dahlia should be cut when flowers are at least 75% open (Armitage and Laushman 2003). Flowers were transported in tap water to a constant temperature room set at 21 °C and a 12 h photoperiod from cool white fluorescent tubes (15-20 $\mu\text{M m}^{-2} \text{sec}^{-1}$). All leaves were removed and stems cut to lengths of 20 cm.

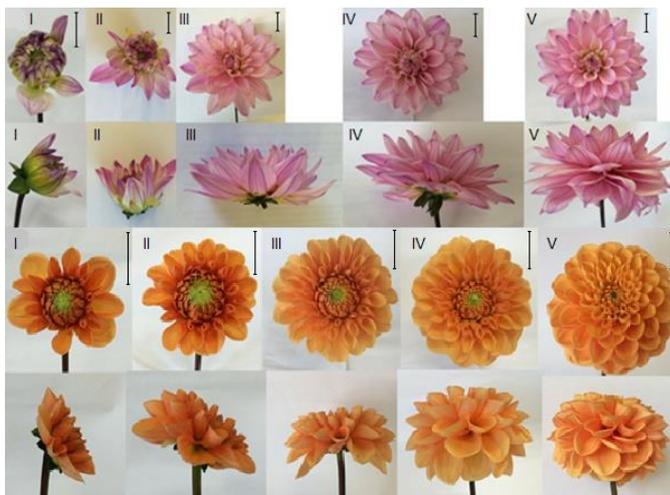


Figure 1. From left to right stages I-V of flower development for the dahlia 'Karma Prospero' (top) and 'Sylvia' (bottom). All scale bars represent 2 cm.

Postharvest treatment

'Karma Prospero' flowers were treated with BA (6-benzylaminopurine; Sigma Aldrich) or STS (sodium thiosulphate; BDH Chemicals, silver nitrate; Sigma Aldrich). Flowers treated with

STS were placed in 10 ml of 4 mM STS (50:50 (v/v) 32 mM Sodium thiosulphate plus 8 mM AgNO₃) for 1 hour before being removed to distilled water in the case of BA treated flowers or with 1% Chrysal Lily preservative in STS treated flowers. BA treated flowers were placed in a fume hood and sprayed with 500 µM BA dissolved in 5% DMSO (dimethyl sulfoxide) or sprayed with distilled water as a control until the entirety of the flower head's surface was covered in the solution. Three whole inflorescence replicates were used for each treatment. To harvest florets for RNA isolation or mass measurement, the diameter of whole flowers was measured and florets of the concentric outer third were considered 'outer' and the inner third 'inner'. For mass measurement five outer florets were harvested from each of the three flower heads and their fresh weight was recorded.

RNA sequencing and de-novo assembly of transcriptome

Florets were removed from stage III inner (3i), stage IV inner (4i), and stage IV outer (4o) flowers of 'Sylvia' (Fig. 1). Three biological replicates were collected from each of these three stages producing nine samples in total. Each biological replicate consisted of nine florets, made up of three florets each from three different flower heads. The first biological replicates for all three stages were harvested during the 2015 season and the second and third biological replicates were harvested during the 2016 season, collecting in two different seasons increased robustness and helped prevent exceptional results that could have been recorded in a single aberrant season. During quality control the single 2015 season biological replicates for each stage were not found to differ from the two 2016 season biological replicates for each stage any more than the 2016 replicates differed from each other. For RNA isolation the RNeasy Plant Mini Kit was used, and RNA extracted according to the kit protocol (QIAGEN, 2016). Samples were stored at -80 °C. All RNA samples were quality tested using a Qubit fluorometer and then sequenced using an Illumina NovaSeq 5000 to produce paired-end reads for each sample. The transcriptome assembly was performed using reads from all nine samples with the Trinity software package (version 2.3.2) with default settings (Grabherr *et al.*, 2011). Low quality reads, bases, and adapter sequences were removed by Trimmomatic (version 0.35) (Bolger, Lohse and Usadel, 2014). The trimmed paired reads of each sample were mapped against the reference transcriptome constructed by the Trinity pipeline using Bowtie2 alignment software (version 2.2.3) (Langmead and Salzberg, 2012). Due to the large number of contigs retrieved at this stage a clustering process was used based on sequence similarity and expression; counts for each sample were recorded into a count table using Corset (version 1.06). Corset was specifically created for *de novo* assemblies to provide 'gene-level' counts which can follow directly into differential gene expression analysis without the need for multiple steps (Davidson & Oshlack, 2014). One of the 3i replicates, from the 2016 season, was removed from further steps due to lack of homology. Blastx alignment queries were performed using the Blast+ 2.2.29 programme. Output parameters provided the top hit for each contig, with an *E*-value cut-off of 1e-5 (Lehnert & Walbot, 2014). Blastx alignments were run against *Arabidopsis thaliana* TAIR10 and TAIR11 (Berardini *et al.*, 2015). Differential expression analysis was carried out using the DeSeq2 package for R statistical software. Relative expression and fold change were then compared between samples. The DeSeq2 output for each comparative list of differentially expressed genes was filtered using Microsoft Excel to contain only those with a q-value (p-adjusted value) < 0.05, and then separated by positive and negative log fold change values.

RESULTS

Flowers treated with BA (Fig. 2b) were visibly less wilted and in better condition compared to control flowers 7d after treatment (Fig. 2a). There was limited wilting of outer florets, but flowers otherwise maintained their morphology and attractiveness. The outer florets of BA treated flowers were significantly heavier ($p < 0.01$) than those of untreated flowers after 7d (Fig. 3). The mean fresh mass of BA treated outer florets was over double that of control florets at 65.2 mg compared to 32.4 mg, 7 d after treatment, this 101% difference compares to a 51% difference between control and 4 mM STS (1 h pulse) & 1% lily food treated florets (Fig. 3). Florets treated with STS showed a significantly greater mass than those of control flowers after 10d ($p < 0.05$; Fig. 3).

The results of the RNA-sequencing showed that the number of reads produced per sample was up to nearly 75M and after analysis it was possible to align the dahlia contigs to over 20,000 *Arabidopsis thaliana* homologs (Table 1). Table 1 shows the number of contigs assembled after the samples were mapped against the reference transcriptome using Bowtie2 alignment software (version 2.2.3), the number of alignments to *A. thaliana* genes after running the Blast+ 2.2.29 programme and the number of differentially expressed contigs produced after use of the DeSeq2 package ($p < 0.05$). Figure 4 shows the percentage of contigs from each sample comparison which were either significantly up or down regulated according to their adjusted p value ($p < 0.05$). The greatest proportion of contigs found to be differentially expressed were found in the 3i vs. 4o comparison, and the fewest in the 3i vs. 4i sample comparison (Fig. 4).

Table 1. A summary of contig number, alignment and significant change by sample comparison

Sample	Contigs	Dahlia contigs with alignments to <i>A. thaliana</i> homologs	Significantly up or down regulated ($p < 0.05$)
3i vs. 4i	92478	46999	2455
3i vs. 4o	122708	109188	22851
4i vs. 4o	122708	109302	17758

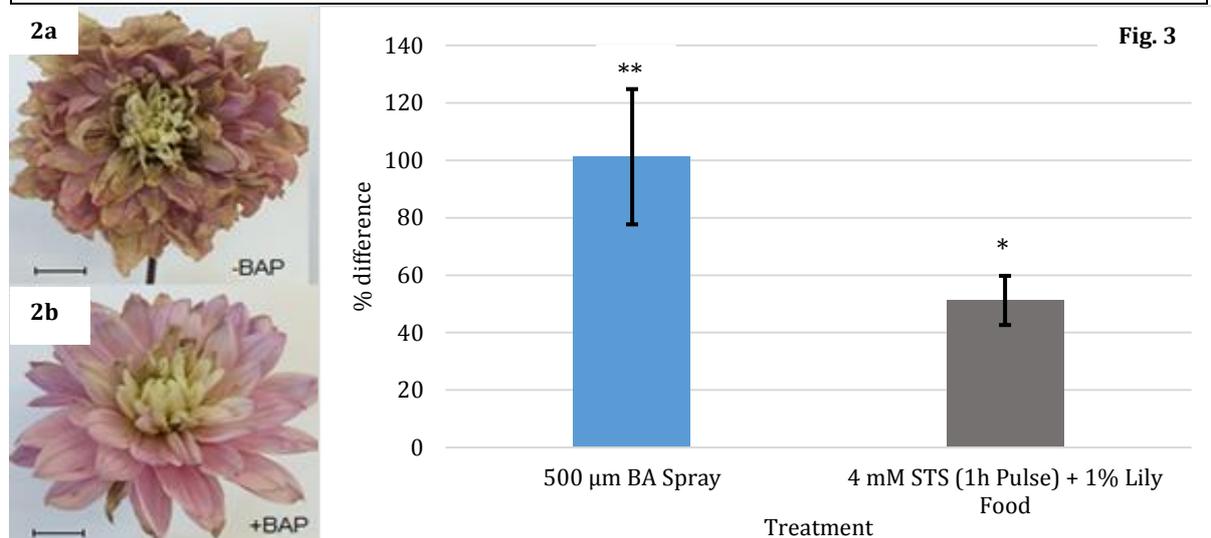


Figure 2. Control 'Karma Prospero' flowers harvested at stage III and held in dH₂O for 7 days (a) flowers treated with 500 µM BA (b). All scale bars represent 2 cm.

Figure 3. Percentage difference in fresh weight of outer florets from 'Karma Prospero' flowers, harvested at stage III (Fig. 1), 7 d after treatment with 500 µM spray of BA (mean + S.E.; n=10) or with 4 mM STS (1h pulse) and 'Lily Chrysal' preservative (1%) (mean + S.E.; n=3). Significant differences to the control (distilled water spray and solution respectively are indicated by * $p < 0.05$, ** $p < 0.01$ (unpaired t-test).

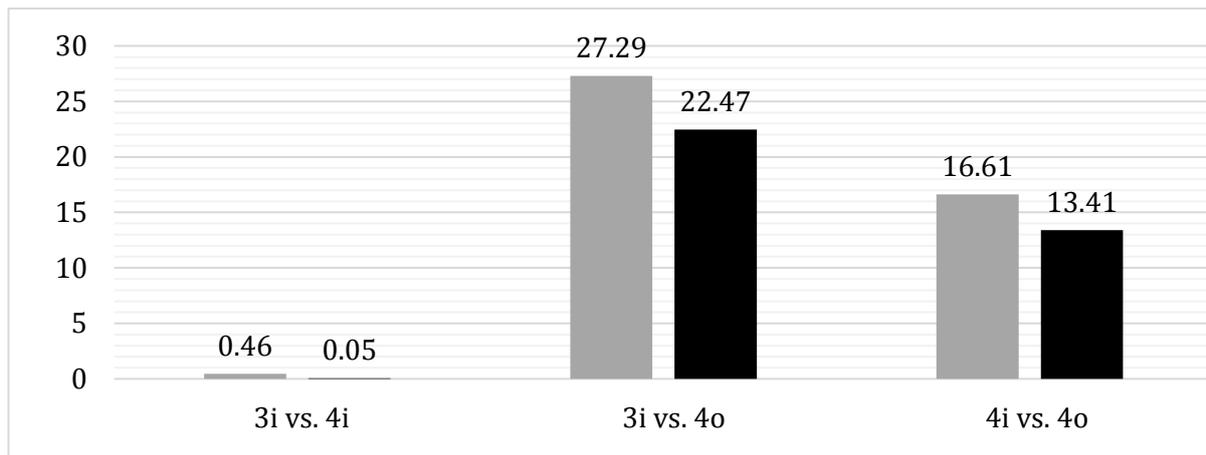


Figure 4. Number of contigs, as a percentage of the total alignments to *A. thaliana*, which were significantly up or down regulated when each of the samples were compared ($p < 0.05$); Grey = upregulated, Black = downregulated.

DISCUSSION

BA-induced improvement in postharvest attractiveness 7d after cutting strongly suggests cytokinins have a role in the control of senescence of at least the dahlia cultivar, 'Karma Prospero' (Fig.2, 3). Such results are consistent with those for other dahlia cultivars (Shimizu-Yumoto and Ichimura, 2013), also using 500 μM BA. However, 50 μM and 100 μM BA were more effective at lengthening vase life compared to 500 μM treatment in other dahlia cultivars (Shimizu-Yumoto and Ichimura, 2013), suggesting that lower concentrations of BA should be tested on 'Karma Prospero'. Moreover 'Kokucho' flowers were treated immediately after being transported, when the outer two whorls of petals were opening, suggesting a stage comparable to between II and III according to our staging (Fig. 1; Shimizu-Yumoto and Ichimura, 2013). Here flowers were harvested at stage III suggesting it may be possible to cut and treat 'Karma Prospero' flowers earlier and perhaps further extend their vase life. STS, an ethylene action inhibitor also slowed down rate of fresh weight loss in 'Karma Prospero' (Fig. 3). This suggests that this cultivar is slightly ethylene sensitive, and that exogenous BA treatment may repress negative effects of ethylene responses.

Further analyses of the transcriptome data may reveal which pathways are critical to maintaining flower quality for a longer period of time. The expression analysis of our RNA-sequencing database will be used to examine which molecular pathways are most up or down regulated in floral tissue, including those related to the signalling or biosynthesis of important phytohormones such as ethylene and cytokinins (Table 1, Figure 4). It is estimated that among plant genomes which have been fully sequenced there are around 20,000 to 40,000 genes (Arabidopsis Genome Initiative, 2000; Matsumoto *et al.*, 2005; Amborella Genome Project, 2013). These estimates are much higher, around 95,000 genes, for the complex hexaploid bread wheat genome, partly due to it being descended from three diploid ancestors (Brenchley *et al.*, 2012). This suggests the octoploid dahlia, the result of hybridisation of two tetraploid wild ancestors, may have a similarly large number of genes (Gatt *et al.*, 1998; Temsch *et al.*, 2008; Sorensen, 1969).

Our annotations to the *A. thaliana* genomic data, which give in excess of 100,000 unique dahlia sequences with homology to *A. thaliana* genes, suggest that this will be a good resource for future studies on dahlia flower senescence on a molecular level (Table 1, Fig.4). The large number of contigs showing differential expression between 4i and 4o compared to 3i and 4i suggests that there are a greater number of gene expression changes during this part of the process. These genes may be linked to the beginning of flower senescence; however, more analysis of the data is required to confirm what process these gene expression changes are related to. Furthermore, there are limitations to this method as multiple unique dahlia loci may be represented by a single *A. thaliana* gene. This is due to *A. thaliana*'s relatively small genome and the dahlia's large and complex genome by comparison. The genomes of Asteraceae members are known to have undergone multiple duplication events in evolutionary history and there is evidence that this has

led to functional divergence within the same gene family, such as *CYCLOIDEA* (Tähtiharju *et al.*, 2012; Chapman *et al.*, 2008). Additionally, *Dahlia variabilis* is thought to be an octoploid, the product of an autoallopolyploidy event when two tetraploid dahlia species hybridised (Schie *et al.*, 2014; Lawrence & Scott-Moncrieff, 1935). It is therefore possible in dahlia that unique contigs which have matched to the same *A. thaliana* gene may be duplicates of a single gene but now have different functions. This has been shown to be the case in floral developmental genes in close relatives of dahlia, including sunflower and *Gerbera*. Studies in these genera found multiple gene duplication events in floral developmental genes such as *CYCLOIDEA*, further suggesting that there is likely to have been similar gene duplication and functional divergence in some similar dahlia genes, making it harder to directly compare to *A. thaliana* (Teeri *et al.*, 2006; Carlson *et al.*, 2011; Chapman *et al.*, 2008; Tähtiharju *et al.*, 2012).

CONCLUSION

From the data presented here we may conclude that dahlia cultivars currently popular in the U.K. market are responsive to either ethylene or exogenous cytokinins and that postharvest treatments may extend vase life of these cultivars, improve their postharvest appearance and help maintain floret mass better than control flowers. De-novo assembly of a dahlia floral transcriptome is possible and expression analysis of these data shows that there are a great number of transcriptomic changes preceding the dahlia flower senescence process. Furthermore, this will present a large source of data for future molecular analysis of dahlia flower senescence.

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