

## Commercial processing of Oriental lilies affects bud opening and metabolic dynamics

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### ABSTRACT

Lilies are a high value cut flower typically producing 4–5 flowers per stem, but the opening of young buds of Oriental hybrid lilies is often affected in cut flowers. Commercial treatment includes harvesting of the stem when the oldest bud is closed and at turning colour, approximately 2 ds before it would open on the plant. Stems are then rehydrated, stored chilled for up to 72 h and transported dry. To understand the effect of commercial treatment on the nutrient status metabolomes were compared throughout bud opening from different positions on the stem. At each developmental stage the metabolomic profile was affected by bud position and commercial treatment. Starch accumulated as long as buds remain closed; upon bud opening starch content declined. Reciprocally, sugar levels rose during flower opening and were affected by edge/ midrib location and commercial treatment. Glucose, fructose and sucrose levels remained higher in opened flowers still on the plant. *AMY2* (amylase) transcript levels rose as did those of two sugar transporters (*MST6* and *SWEET7*). Commercial processing therefore impacts on the metabolome and the ability to accumulate sugars in the opening flower bud. Commercial treatment delayed bud opening and the effect was dependent on the position of the bud on the stem. However, it had little impact on the rate of cell expansion during flower opening. Cell expansion in the different areas of the adaxial epidermis was unaffected by the commercial treatment. Furthermore, edge and adaxial tepal cells expanded faster during opening. Expression of cell expansion related genes (*EXPA1* and *LOPIP1*) fell as flowers opened. This differential cell expansion in the tepal sectors could underpin the transition of a convex to a concave tepal shape during opening. In conclusion, commercial processing mainly affects the early stages of bud opening. Sugar and metabolite accumulation is compromised by commercial treatment, but this did not affect the capacity for cell expansion in the tepal. Furthermore, our data indicate that differential cell expansion in the different sectors of the tepals is important in lily flower opening, and that this is associated with starch breakdown and sugar accumulation.

### 1. Introduction

Lilies are an economically important ornamental crop consisting of Oriental and Asiatic hybrid varieties as well as *Lilium longiflorum* hybrids. Most varieties produce stems with 4–5 buds which are harvested at a defined stage of development to optimise flower opening in the vase. The stage of harvest is critical to ensure that they do not open too soon, resulting in a short consumer vase life and susceptibility to mechanical damage in the supply chain. Buds open sequentially up the stem, thus the stage of maturity at harvest is optimised to ensure maximal number

of opening buds; however the youngest smallest buds may fail to open once detached from the plant. Bud opening can therefore also be a postharvest problem since buds on stems that are harvested too early can fail to bloom. This has been linked to nutrient competition between buds on the stem, exacerbated by insufficient nutrient availability (Van Doorn and Van Meeteren 2003) and low-light conditions during transport, storage and post-sale. Cold/dark treatment is commonly used commercially for cut flowers to slow down their metabolism and lower their respiration rate, retaining product quality for longer (Galati et al. 2020), however low light conditions can be responsible for severely restricting

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photosynthesis of buds, thus limiting nutrients. Stems are then transported dry in boxes, under low light, causing additional dehydration of the buds. This exacerbates cold stress effects and can reduce the longevity of cut flowers (Rudnicki et al. 1991; Wagstaff et al. 2010).

Flower opening is a highly dynamic process. Lily tepals change shape from closed buds, where they are curved to face the reproductive organs, to fully open flowers, where they reflex in the opposite direction. In some varieties opening is also associated with rippling of the tepal edges. Opening also includes movement at the base of the flower causing the tepals to move apart from each other (Liang and Mahadevan, 2011). These changes in shape require rapid differential growth of the tepals. The speed of lily flower opening, which can be as short as 4 h from the mature bud to fully open flower (Bieleski et al. 2000), is consistent with this being driven by cell expansion as is the case in other species (e.g. rose, Yamada et al. 2009). Indeed, in *L. longiflorum* tepals, cell division appears essentially to cease when they are a third of their mature length (Gould and Lord, 1989) and there is only a very small increase in the number of epidermal and parenchymal cells over flower development and opening in Oriental lilies (Watanabe et al. 2022).

Based on tepal measurements and epidermal cell diameter in the inner and outer epidermis of Asiatic lilies it was suggested that cells might expand faster on the adaxial face compared to the abaxial face to effect a global change from a concave to a convex shape (Bieleski et al. 2000). Further measurements of opening flowers in an Oriental hybrid lily combined with mathematical modelling suggested that cells expand faster at tepal edges than the midrib, which could cause the distinctive saddle shape and rippled tepal edges (Liang and Mahadevan, 2011). However, there is a lack of data on localised changes in cell size across all dimensions of the tepal, and how these might contribute to tepal expansion and flower opening. Intracellular and cell-wall morphology, composition and structure also change over flower opening with differential changes in cell morphology in adaxial compared to abaxial sides of the outer tepal prior to flower opening (Zhang et al. 2021). This may be due to differences in cell wall loosening since genes coding for cell wall loosening enzymes: expansins and xyloglucan endotransglucosylase/hydrolases were expressed significantly more in tepal adaxial than abaxial epidermal cells, correlating with a greater rate of cell expansion in adaxial cells (Watanabe et al. 2022). Zhang et al. (2021) also suggested that the twisting and rolling out of tepal edges is driven by the adaxial epidermal cells at the tepal edges having a smaller cell wall surface area (defined by these authors as fewer wrinkles) than internal cells, and a higher water absorption capacity, leading to their greater overall expansion.

There is a positive correlation between lily bud development (length) and tepal sucrose content, with the opening flower containing the most sugars, comprising glucose, fructose and sucrose (Clément et al. 1996; Van der Meulen-Muisers et al., 2001). Indeed, supplementing LA hybrid lilies (cv. Courier) with additional 1 % sucrose accelerated flower opening by 2.4 ds on average (Arrom and Munne-Bosch 2012), while addition of 2 % sucrose in the vase solution of Oriental lilies (cv. Stargazer) slightly improved the percentage of buds opening compared to controls. Indeed, some controls could not complete full flower opening (Han, 2003). Treatment of excised Asiatic tepal segments with the phosphoglyceric acid (PGA) which promotes starch synthesis and  $\alpha$ -amylase inhibitor protein (AIP) which inhibits starch breakdown caused a reduction in their expansion (Bieleski et al. 2000), suggesting that starch breakdown to sugars may be needed for tepal expansion. Both glucose and fructose were found to rise during Oriental lily bud development and flower opening while sucrose content fell (Watanabe et al., 2022).

In addition to starch breakdown, sugars are also transported to developing buds from other organs: phloem loading and unloading is an important regulatory step which can influence the sucrose and starch content throughout development of flower buds. There are two major mechanisms for phloem loading, dependent on species and anatomy: apoplastic, and symplastic (Zhang and Turgeon, 2018). Apoplastic

phloem loading involves uptake of sucrose from the apoplast by active transport. Conversion of sucrose into monosaccharides occurs in the extracellular space by cell wall invertases (CWINVs) and uptake into tepal cells is then by hexose/monosaccharide transporters (HXTs/ MSTs) (Rennie and Turgeon, 2009). Symplastic loading is either driven passively by creating a high sucrose gradient from mesophyll to phloem, or by conversion of sucrose to oligosaccharides (polymer trapping). Sucrose from the phloem moves into companion cells (CC) and then into tepal cells via sucrose transporter proteins (STPs/ SUTs and SWEETs), entering the cell as sucrose (Durand et al. 2018). Cell expansion also requires water uptake and cell wall remodelling. Aquaporins are cell membrane water transporters allowing free water uptake into the cell along the osmotic gradient. A PIP aquaporin (PIP2) has been functionally characterized and identified as responsible for cell expansion in rose petals (Ma et al. 2008). Expansins (EXPs) and xyloglucan endotransglucosylases/hydrolases (XTHs) are important in cell, tissue and organ growth due to their cell wall-loosening and remodelling properties (Ishida and Yokoyama, 2022). A gene coding for an expansin (EXPA) showed expression which strongly correlated with flower opening in rose (Yamada et al. 2009) and lily (Watanabe et al. 2022).

Here, new data are presented on the effects of commercial treatment of Oriental lily stems in relation to bud opening, the typical commercial treatment comprising cutting of stems, treatments with preservatives, cold storage and dry transport. Whilst commercial treatment affects rate of bud opening, carbohydrate and metabolome dynamics, the overall cellular mechanism of flower opening appears unaffected. A model for bud opening is presented based on measurement of localised changes in cell expansion across the tepal, changes in metabolomic profiles, sugar content and the expression of key genes involved in sugar transport and cell expansion.

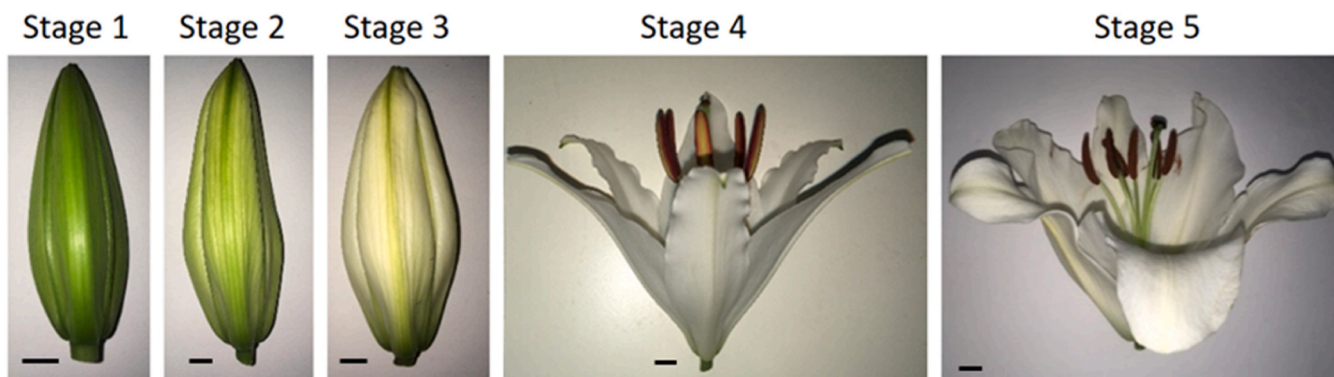
## 2. Methods

### 2.1. Lily growth, harvesting and postharvest conditions

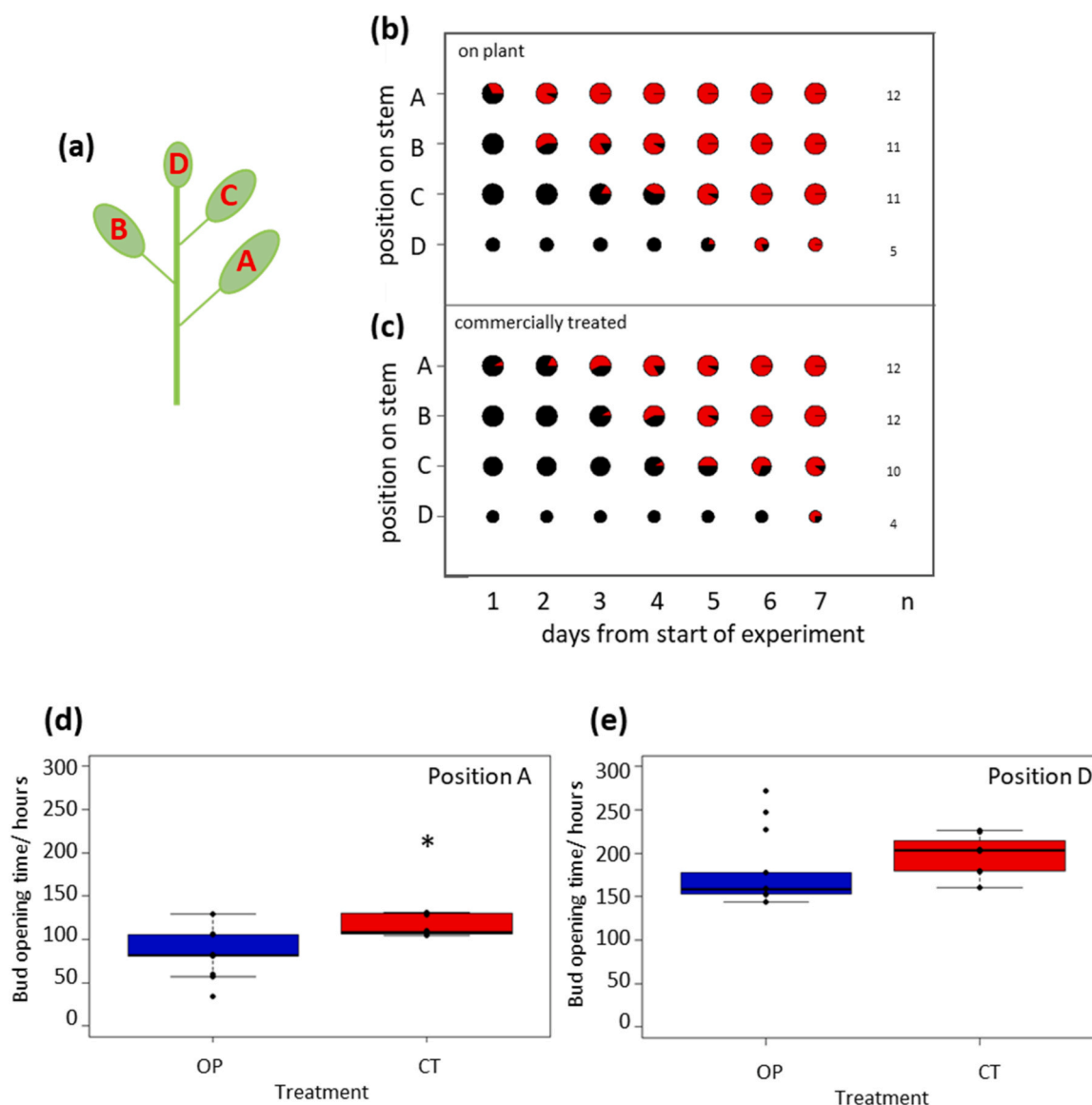
For all experiments Lily stems were grown to maturity under greenhouse conditions at E.M. Cole Farms Ltd. (variable temperature average 18–20 °C, no additional lighting) in crates (approximately 60 × 40 × 20 cm, 12–15 bulbs per crate) prior to harvest, unless otherwise stated. Varieties used included Oriental hybrid lily cvs. ‘Ascot’, ‘Tisento’, and LA hybrid lily cv. ‘Eyeliner’. Stems were harvested in the morning 07:00–12:00 dependent on the size of the largest bud and the ambient temperature (as per current commercial guidelines). Bud maturity was assessed based on the size and appearance for each variety where buds of position A were at approximately Stage 2 of development (Figs. 1 and 2). Stems were rehydrated according to standard commercial treatment in FloraLife Express Clear ULTRA 200 (1:200 water) with 30 stems per 2 L nutrient solution for up to 72 h at 4 °C in dark room conditions prior to packaging and dry transport to Cardiff University in cardboard boxes (approximately 5 h). Cut lily stems were maintained before and during experiments in growth room conditions (up to 30 stems were rehydrated and stored in 2 L Chrysal Clear Liliun & Alstroemeria solution in tap water (made up as directed – 1 sachet per 1 L tap water) at 21 °C, 16 h light (155  $\mu\text{mol m}^{-2} \text{s}^{-1}$  metal halide bulbs):8 h dark unless otherwise stated. Vase solution was regularly topped up as necessary to prevent containers from drying out. Commercial solutions for flower stems typically contain sugars and biocidal components to reduce microbial growth. Buds were graded by stage of development (Fig. 1).

### 2.2. Analysis of flower opening time

Whole lily plants (Oriental cv. Tisento) in crates, cut stems, or individual buds were carefully selected for equal stages of development based on bud length, measured using an electronic digital caliper (Precision Gold, Maplin Electronics UK) at the start of the experiment. Each bud was labelled with a unique coloured label to identify position on



**Fig. 1. Stages of Oriental lily cv. Tisento flower development.** Scalebar = 1 cm. Stage1, approximately 3 d prior to opening (small green immature bud); Stage 2, approximately 2 d prior to opening (turning colour, larger bud); Stage 3, approximately 1 d prior to opening (mature bud, almost fully white); Stage 4, half open bud, prior to anther dehiscence; Stage 5, fully open mature flower. Scalebar = 1 cm.



**Fig. 2. Effect of commercial treatment on bud opening in Oriental lilies ‘Tisento’ and ‘Pacific Ocean’** (a) position of buds on stem with bud A the oldest and bud D the youngest; pie charts of on-plant (b) and commercially treated (c) ‘Tisento’ stems. Red indicates the proportion of total buds of each position which were open at each day from experimental start until day 7. Size of pie indicates number of buds; n indicates the total number of buds at each position used in the experiment. (d) and (e) number of hours from the start of the experiment to start of bud opening in ‘Pacific Ocean’ stems in (d) position A buds and (e) position D buds. Data points are shown in black; asterisks indicate statistical significance (Student’s T-test;  $p < 0.05$ ).

stem and stem identity. For cut stems, up to 6 stems were arranged in each bucket (containing 2 L FloraLife solution made up as directed). Plants and flowers were set up facing timelapse cameras (Logitech C270 HD, Wansview 1080p). These were programmed with a Raspberry pi using the Python program fswebcam to take photographs hourly. Photographs were analysed by hand to identify date and time of opening for each individual bud. Bud opening time data (number of days from start of experiment) was analysed using a Generalized Linear Mixed Model (GLMM) in RStudio (version 1.3.1093) to identify significant differences between on-plant and commercially treated stems with respect to position on stem.

A second similar experiment was set up using the same equipment to measure bud opening time in Oriental lily cv. Pacific Ocean. On-plant and commercially treated stems (as described above) were set up in greenhouse conditions and cameras were positioned as above to take photos every 30 min. A dim green light (Phillips Living Colors 69143/87/PU LED Lamp, ENUOLI green neon light) was used at night to allow continuous 24 h photography whilst minimising the effect of artificial light on the plants, which was not possible in the previous experiment. Although plants are able to perceive green light it has less impact on growth and flowering than red or blue wavelengths (Battile et al. 2020). Bud opening time was analysed using one-way ANOVA in RStudio (version 1.3.1093) to identify differences in hours taken to open between on-plant and commercially treated buds.

### 2.3. Analysis of tepal epidermal pavement cell growth

Commercially treated Oriental hybrid lily stems with white flowers (cv. unknown) were bought from a retailer and stored in growth room conditions until required. Flowers were graded from Stage 1–5 and a single representative flower for each stage was selected. In a second experiment, flowers from commercially treated cv. Pacific Ocean were used with three biological replicates at stages 1, 3 and 5. Tepal material was prepared for microscopy in 100 % ethanol overnight, then 10 mm<sup>2</sup> sections from the tip, middle and base, proximal to the tepal edge and midrib of outer and inner tepals were incubated in lactic acid until the tissue turned clear. Phase contrast light microscopy (Axio Imager M1 (Zeiss) and bScope BS.1153-EPLi (Euromex)) was used to image adaxial epidermal pavement cells. Cell area was measured using imageJ Fiji software. Fold change in epidermal pavement area between Stage 1 and 5 was calculated using the calculation below for each section:

(mean epidermal pavement cell area at Stage 5 – mean epidermal pavement cell area at Stage 1)

mean epidermal pavement cell area at Stage 1

A GLMM was carried out in RStudio (version 1.3.1093) to identify significant differences between cell area growth in different parts of the tepal during development.

### 2.4. Qualitative and quantitative assessment of tepal starch content

Oriental lily cv. Ascot was grown in E.M. Cole Farms Ltd. greenhouse conditions as described above. ‘on-plant’ (OP) samples were collected directly from growing stems, while ‘commercially treated’ (CT) samples were from stems subjected to the commercial treatment described above. Three individual flowers were harvested at Stages 1, 3, and 5 from the lowest bud on the stem (position A, Fig. 2a). For the qualitative assay one inner and one outer tepal from each flower were immediately submerged in hot 80 % ethanol and incubated at 80 °C for 30 minutes. Tepals were rinsed in distilled water and incubated in Lugol solution (Sigma-Aldrich, London) at room temperature for 15 minutes. Tepals were rinsed in distilled water and photographed.

For starch quantification the lowest bud (position A) and third lowest bud (position C) from stems consisting of 4–5 buds, were harvested at Stages 1, 3 and 5 for OP and CT treatments. Tepals from each flower

(three replicates) were split into midrib and edge sections, flash frozen in liquid nitrogen and fragmented with a mortar and pestle. Starch was quantified according to Smith and Zeeman (2006). Soluble sugars were extracted from 150 mg of tepal material using 80 % ethanol (5 mL) at 100 °C for 3 min, then centrifuged at 8000 g for 5 min. The insoluble pellet was air dried and ground to a paste by pestle and mortar with 5 mL dH<sub>2</sub>O. Samples (0.5 mL) were incubated for 30 minutes at 100 °C and autoclaved for 15 min to gelatinize starch particles. Breakdown of starch granules was confirmed by Lugol staining light microscopy (bScope BS.1153-EPLi (Euromex)).

Statistically significant differences with regard to commercial treatment and location on tepal were evaluated using one way ANOVA and post hoc Tukey test for the first two comparisons and using an independent T-test/ Mann-Whitney U test for the last comparison. All statistical analyses were carried out in RStudio (version 1.3.1093).

### 2.5. Metabolite fingerprinting and quantitative sugar analysis

Metabolites were extracted from flash frozen and fragmented lily tepal material (Oriental lily cv. Ascot; 50 mg) using chloroform/methanol/water (1:3:1). Three biological and two technical replicates were used, representing three individual flowers. Following centrifugation, the supernatant from the extraction was used in further analysis. Flow injection electrospray high resolution mass spectroscopy (FIE-HRMS) was carried out on an Exactive HCD mass analyser linked to an Accela UHPLC system (Thermo-Scientific) which produced metabolite fingerprints in positive and negative ionisation mode in a single run. Supernatant extract (60 µL) was directly injected to a flow of 100 µL min<sup>-1</sup> methanol: water (70: 30, v/v). Ion intensities were measured between 50 and 1000 *m/z* at a resolution setting of 100,000 (at *m/z* 200) with a mass accuracy of 3 (±2) ppm for 3.5 mins. ESI source parameters were set according to the manufacturer’s recommendations. Raw files were exported as .raw files, converted to *mzML* files using Proteowizard, and mass aligned using R-package *binneR*. Mass spectra around the apex of the infusion peak were combined in a single intensity matrix for each ion mode. Data from the intensity matrix was log-transformed before further statistical analysis. Spectral processing, data mining, and feature selection was performed using Random Forest in R package FIEms-pro as reported previously (Enot et al. 2008; Finch et al., 2022).

Data analysis of full profiles from FIE-HRMS was carried out using PerMANOVA, Canonical Analysis of Principal component (CAP) analysis, and Random Forest in RStudio (randomForest, vegan packages, RStudio version 1.3.1093 (Liaw and Wiener, 2002; Oksanen et al., 2022)) to identify significant differences between whole metabolic profiles (Breiman, 2001; Anderson and Willis, 2003). Compounds which were most important in the classifications for each of the Random Forest analyses were putatively identified using the DIMEdb database (Draper et al., 2009) using the *m/z* ratio and ionisation type, as well as the ion type information if available for each compound. The compounds suggested by the database were screened for accuracy to the measured *m/z* ratio and for reasonable presence in plant tissue, and the most likely candidate was taken as the ‘putatively identified compound’.

Gas chromatography- time of flight mass spectrometry (GC-tofMS) was carried out using the same material to identify glucose, fructose and sucrose content, alongside other selected compounds. Supernatant extract (5 µL) and 5, 20 and 30 µL of the carbohydrate standards were used. An internal standard (25 µL L-threo-tertbutylserine) was added. Carbonyl moieties of metabolites were protected by methoximation using 10 µL 20 mg mL<sup>-1</sup> solution of methoxyamine hydrochloride (Fluka) at 30 °C for 90 mins. Metabolites were then derivatised with 20 µL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, M and N) at 37 °C for 30 min. Derivatised material (1 µL) was injected split-less into a Leco Pegasus III GC-tofMS (St. Joseph, USA), comprising a Focus autosampler (Anatune) and anAgilent 6890 N gas chromatograph equipped with DB5-MS column (20 m x 0.25 mm ID x 0.25 µm film). Injector, transferline, and ion source temperature were set to 250 °C,

240 °C, and 230 °C, respectively. The helium flow rate was 1.4 mL min<sup>-1</sup>. After 1 min at 80 °C, oven temperature was increased by 30 °C min<sup>-1</sup> to 330 °C, held at 330 °C for 3 min before cooled to 80 °C. Automated deconvolution and peak finding was performed using Chroma-Tof software (Leco, St. Joseph, USA) and peak alignment was carried out in MATLAB (V7.5.0, The MathWorks).

Data analysis of full profiles was carried out using Permanova, Canonical Analysis of Principal component (CAP) analysis, and Random Forest in RStudio (version 1.3.1093). Statistical analysis of differences in individual tepal sugar content (glucose (two anomers), fructose (two anomers), sucrose (one isomer)) was carried out using the area under the peak measurements from GC-MS data, using the peaks at retention times confirmed by internal standards of fructose, glucose and sucrose. Differences were evaluated using one way ANOVA and post hoc Tukey test in RStudio (version 1.3.1093).

## 2.6. RNA extraction and real time qPCR

RNA was extracted from 150 mg ground frozen Oriental lily cv. Tisento tepals (three replicates) using a CTAB based method (Gambino et al., 2008). DNase treatment was carried out using the Turbo DNA-free kit (Invitrogen) according to manufacturer's instructions and cDNA was synthesised from DNase RNA using the GoScript™ Reverse Transcription kit with Oligo-dT (Promega). DNase treatment and cDNA synthesis efficacy were checked by PCR with AUX1 primers (Supplementary Table 1). Real time qPCR reactions (20 µL) contained 10 µL 2x qPCR BIO SyGreen Blue Mix Lo-ROX master mix (PCR Biosystems), 0.4 µL of each forward and reverse primers (10 mM), and cDNA which had been diluted appropriately. Primers for the reference gene EF1 were used to normalise cDNA concentrations and calculate the relative expression of other genes of interest (all primers are listed in Supplementary Table 1). A LightCycler® 96 instrument (Roche) was used with 95 °C for 5 s, 35 cycles at 30 s at 95 °C, 30 s at an annealing temperature depending on primers used, and 30 s at 72 °C for extension. A melting curve was run to check primer specificity (95 °C for 60 s, 55 °C for 30 s, 95 °C for 1 s). Relative gene expression was quantified using the delta-delta Ct (2<sup>-DDCt</sup>) method (Livak and Schmittgen, 2001) at Stages 1–5 in commercially treated 'Tisento' tepals. A one-way ANOVA with post-hoc Tukey test or Kruskal-Wallis statistical test with post-hoc Wilcoxon rank-sum was used as appropriate to identify significant differences in relative gene expression between conditions (dependent on normal distribution of the data).

## 3. Results

### 3.1. Flower opening in commercially treated stems is delayed compared to on-plant controls and is affected by position on stem

Flower opening was compared between Oriental lily buds on the plant and those on stems that had been harvested and subjected to the commercial treatment including cold and dark storage for 72 h. Stems were harvested with position A buds at Stage 2 of development. Bud opening on-plant was sequential with approximately 1 d between each of the bud positions A to C, and 2 d between bud position C and D (Fig. 2a,b). Commercial treatment delayed flower opening (Fig. 2c).

Position A buds still started to open on day 1 of the experiment, positions B and C were delayed by one day, and position D buds were delayed by 2 d. Indeed, even on day 7 only 9 out of 10 position C buds, and three of the four position D buds had opened when stems were commercially treated, whereas all position C and D buds on the plant were open by day 7, showing the delaying effects of the commercial treatment. However, bud position was also an important factor as all position A and B buds opened by day 7 even on commercially treated stems.

A second experiment comparing on plant and commercially treated bud opening time in a similar cultivar ('Pacific Ocean') showed that

while commercial treatment had a significant delaying effect on bud opening time in position A buds (Fig. 2d;  $p < 0.05$ ), it did not for position D buds (Fig. 2e). This confirms the interaction between bud position and commercial treatment although the specific effects are also cultivar dependent.

### 3.2. Flower opening is associated with differential cell expansion of epidermal pavement cells across the tepal

Changes in cell size across all three dimensions of Oriental lily outer tepals in cut stems were assessed to test models proposed for flower opening. Overall, stage of development, both position (top, middle or base) and location (midrib or edge), and surface (adaxial or abaxial) all showed a significant effect on cell size (Supplementary Table 2). Moreover, there were interactions between stage and position, stage and surface, and position, location and surface ( $P < 0.001$ ). Pavement cell area increased during bud opening (Fig. 3a; Supplementary Table 2). However, analysing local changes in cell size between Stage 1 and Stage 5 reveals that the increase is not uniform (Fig. 3b-d). Abaxial cells are significantly larger than adaxial cells at Stages 1 and 2, but the rate of increase is faster for adaxial cells (Fig. 3b). Midrib cell area was significantly larger than edge cell area throughout bud development, however the rate of increase was not significantly different (Fig. 3c). Comparing longitudinal position shows that while cell area at the base and tip remains low throughout development compared to the other sectors, cell area in the mid sector increases dramatically between Stages 4 and 5 as the flower opens fully (Fig. 3d).

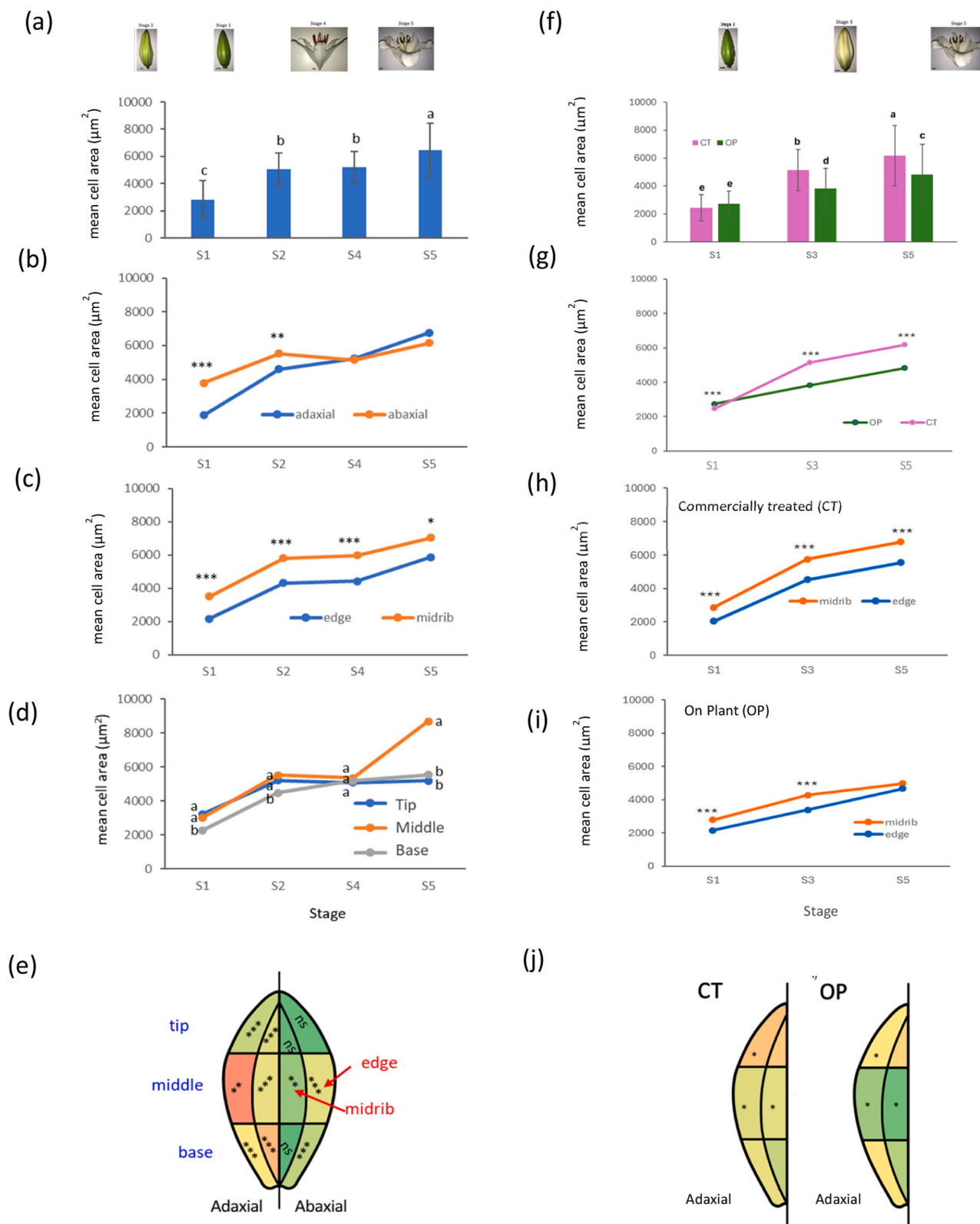
When individual sectors were assessed separately the greatest increase in cell area between Stage 1 and 5 was in the middle adaxial edge cells where there was a 6-fold increase (Fig. 3e), and there was an almost 5-fold increase in cell area in the basal adaxial midrib cells. Cells from three abaxial sectors showed no significant expansion. Thus, localised cell expansion could be an important contributor to tepal shape change as buds open in this variety of lily.

### 3.3. Commercial stem treatment did not have major effects on localised cell area expansion

To assess if cell area changes during bud development are affected by commercial treatment, the same analysis was performed with Oriental lily 'Pacific Ocean' buds. Cell size increased progressively under both treatments but cells from commercially treated (CT) buds were larger at Stage 3 and Stage 5 though not at Stage 1 compared to on-plant (OP) buds (Fig. 3f). There was an interaction between stage, treatment, position, and location, although not between stage and location or stage, position and location. At Stage 1, CT bud cells were significantly smaller than OP bud cells, however at later stages of development CT bud cells were larger than the OP cells at the same stage (Fig. 3g). However, CT and OP, midrib and edge cells expanded in a similar way, with midrib cells consistently larger than edge cells (Fig. 3h and i). In 'Pacific Ocean' again there were clear localised differences in cell expansion across the tepal and these were broadly consistent between buds from CT and OP treated stems (Fig. 3j) although the specific high expansion rate in the middle position, adaxial edge segment was not seen.

### 3.4. Bud position on the stem influences the metabolome throughout development and opening

To assess if the effects seen in flower opening and cell expansion due to tepal sector, position on stem and treatment might reflect metabolic differences, global metabolic changes were analysed by flow infusion electrospray-high resolution mass spectrometry (FIE-HRMS) comparing flowers that developed on the plant, and off the plants subjected to commercial treatment. Given the differences in cell expansion across different sectors of the tepal, tepals were also divided into edge and midrib sectors. Moreover, given the differences across bud position,



**Fig. 3.** Epidermal pavement cell growth in outer Oriental lily tepals over bud development and opening in: (a-e) commercially treated cut stems of Oriental lily (unknown cv.); (f-j) comparison of commercially treated cut stems and on plant of Oriental lily ‘Pacific Ocean’. Mean cell area at each stage of bud development (a and f) across whole tepal (n = 72–360); (b) between adaxial and abaxial cells (n = 36), (g) between commercially treated and on plant (n=1080), (c, h and i) between midrib and edge cells (n = 36–540) in commercially treated (c, h) and on plant (i); (d) amongst top, middle and base (n = 24–720); (e and j) Fold change of cell area (Stage 5 vs. Stage 1) of different sectors of the tepal colour-coded to reflect magnitude of cell size change: (e) including position, location and surface (j) including only adaxial surface comparing commercially treated (CT) and on plant (OP); Asterisks indicate significant differences at each stage of development, (\*\*\* P < 0.0001, \*\* P < 0.001, \* P < 0.05) based on a t-test; different letters indicate significant differences at each stage of development based on a Kruskal-Wallis test with Bonferroni P-value adjustment.

buds at position A and C were compared. Canonical Analysis of Principal coordinates (CAP) was used to assess the similarity of metabolic profiles. Treatment and location on tepal were well discriminated with correct classifications of 100 % (Supplementary Figure 1). However, combining tepal sectors, bud position and treatments, Stage 1 of development was clearly separated from Stages 3 and 5 but these were not clearly discriminated from each other. Position on stem was also not clearly discriminated by the metabolome data. PerMANOVA was used to test interactions between the different variables, showing significant differences between the metabolomes with respect to all variables (treatment ( $R^2$  0.078,  $p < 0.05$ ), stage of development ( $R^2$  0.287,  $p < 0.05$ ), position on stem ( $R^2$  0.0329,  $p < 0.05$ ), and location on tepal ( $R^2$  0.160,  $p < 0.05$ ), with a significant interaction between treatment and stage of development ( $R^2$  0.0446,  $p < 0.05$ ).

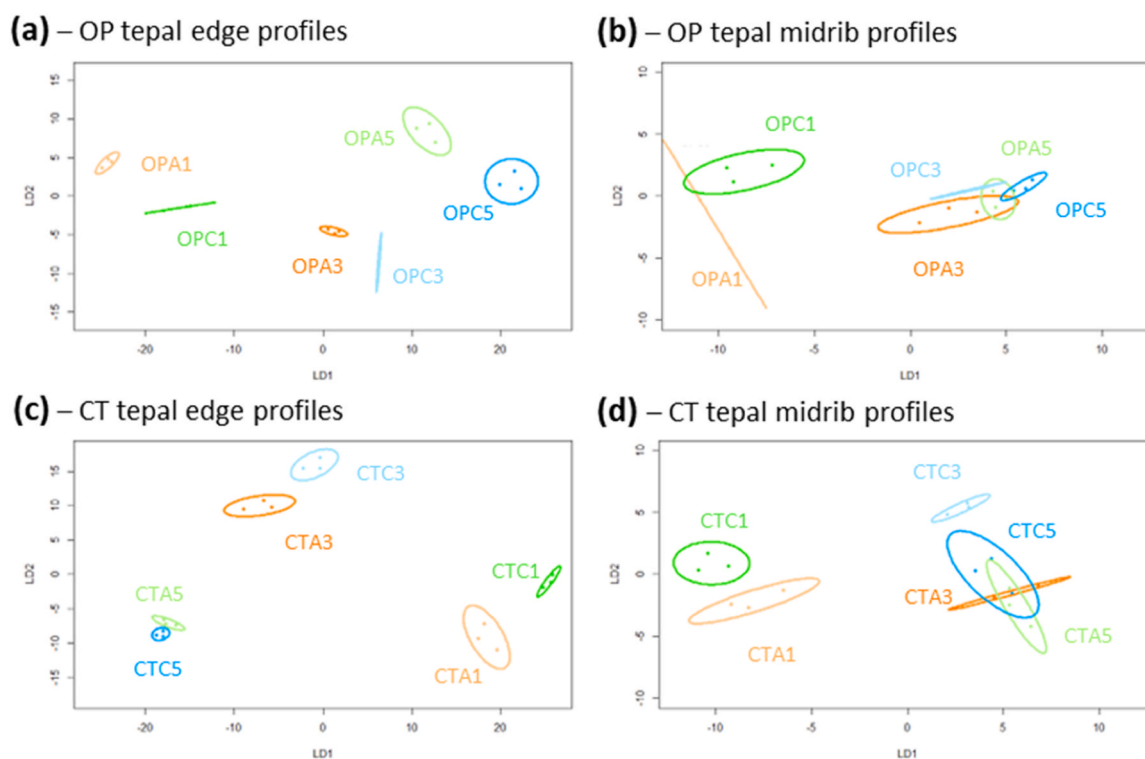
Considering only the tepal edge, on-plant metabolomic profiles showed good discrimination both regarding stage of development and position on stem, (Fig. 4a). On-plant tepal midrib profiles showed much poorer discrimination: At Stage 1 profiles did not discriminate between Position A and C tepals but did discriminate from the rest. At stage 3 position A and C profiles were discriminated but Stage 5 profiles overlapped at the 95 % confidence interval with each other and with the position A stage 3 profile (Fig. 4b). In the commercially treated stems, discrimination between position A and position C buds in the tepal edges was as good as the on-plant stems at Stages 1 and 3, but by the open flower (Stage 5) they were much more similar (Fig. 4c). Midrib tepal profiles from commercially treated stems showed better discrimination of bud position at Stage 1 than those on-plant but there was a similar overlap in profile amongst the two Stage 5 profiles and the Stage 3 position A profile (Fig. 4d).

Random Forest analysis was applied to identify the most discriminatory specific metabolites whose relative abundance was most discriminatory in relation to the different variables tested. This is based on the “mean decrease accuracy” measure that reports on the top 20

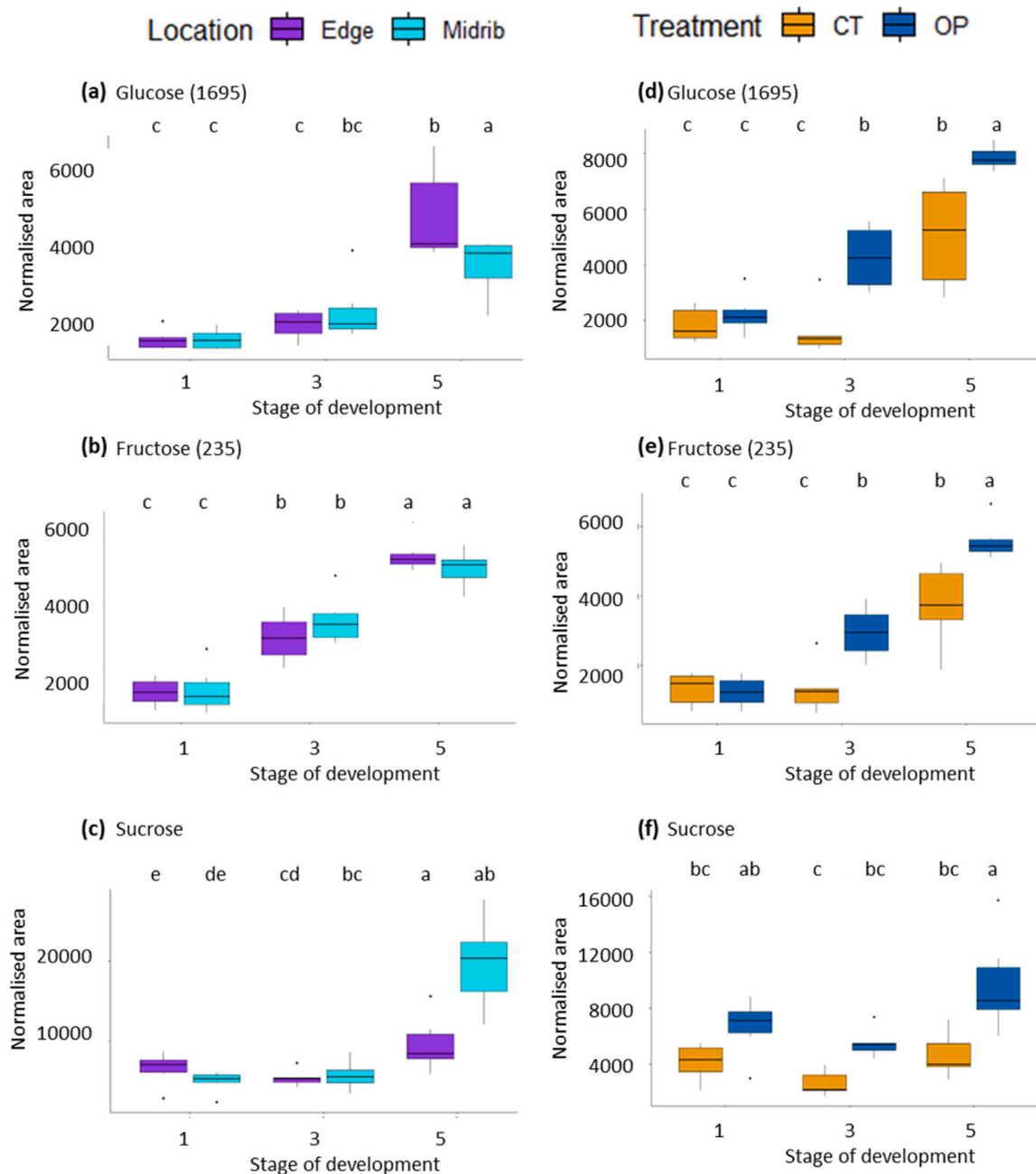
compounds that are most important in classifying the metabolite profiles (Supplementary Table 3). It was only possible to putatively identify very few of the top 20 compounds found in the FIE-HRMS analysis due to a lack of complete databases; however, included in the top eight discriminators for treatment were three phenylpropanoids, a sesquiterpene, a naphthalene, a sulphur-containing compound and an antioxidant compound all of which were more abundant in the commercially treated tepals compared to the on-plant controls at all three stages of development tested. In contrast, a monoterpenoid (genipin), was more abundant in the on-plant control tepals (Supplementary Fig. 2). Amongst the compounds putatively identified as differing most in relative abundance between the edge and midrib were two phenolic and one flavonoid compound that were more abundant in the midrib at all stages of development, and a flavone that was more abundant in the tepal edge sector (Supplementary Fig 3; Supplementary Table 3). Eight of the 20 most discriminatory metabolites that differed most in abundance across the two bud positions based on the Random Forest analysis were phospholipids all of which were higher in abundance in buds from position A compared to position C. Other compounds included putative flavonoids and glycerides (Supplementary Fig 4; Supplementary Table 3).

### 3.5. Tepal glucose, fructose and sucrose content is affected by treatment and differs in tepal sectors throughout bud development

Given the global metabolic differences, changes in sugars were analysed in more detail as these may contribute to cell expansion through an osmotic or nutritional effect. Tepal glucose, fructose and sucrose content was measured in on-plant flowers at 3 stages of development (Stages 1, 3, 5). Content in edge and midrib sections was compared as localised areas of these tepal sectors differed in their rates of cell expansion. All sugars increased significantly between Stages 1/3 and 5, correlating with flower opening (Fig. 5). Tepal glucose was



**Fig. 4.** Canonical Analysis of Principal coordinates based on lily tepal midrib and edge metabolomes collected using FIE-HRMS. (a) On plant (OP) edge profiles, (b) OP midrib profiles, (c) Commercially treated (CT) edge profiles and (d) CT midrib profiles. The models are plotted using the first two linear discriminants and each ellipse represents 95 % confidence interval. Percentage of correct classifications was (A) 94.4 % ( $p < 0.05$ ,  $n=3$ ), (B) 94.4 % ( $p < 0.05$ ,  $n=3$ ), (C) 88.2 % ( $p < 0.05$ ,  $n=3$ ), (D) 83.3 % ( $p < 0.05$ ,  $n=3$ ).



**Fig. 5.** GC-MS analysis of tepal samples quantifying relative tepal sugar content. (a,d) glucose isomer 1695 (b,e) fructose isomer 235 and (c,f) sucrose, comparing edge and midrib sections in flowers on plant (a-c), and tepal edge section content in on-plant (OP) and commercially treated (CT) flowers (d-f). Different letters indicate significant differences based on a two-way ANOVA with Post-hoc Tukey test ( $P < 0.05$ ,  $n=3$ ).

significantly higher in edge sections than midrib at Stage 5 (Fig. 5a), while tepal sucrose was slightly (but not significantly) higher in midrib sections in open flowers (Stage 5, Fig. 5c). Fructose increased with bud development but there was no difference in its concentration between tepal edge and midrib (Fig. 5b).

At Stage 1, glucose content was equal between on-plant and commercially treated samples (Fig. 5d) (considering midrib and edge together) but at Stage 3 and 5 it was significantly higher in on-plant samples compared to commercially treated samples (Fig. 5d). Tepal fructose content showed a very similar dynamics to glucose, showing a significant increase in content between Stages 3 and 5 (Fig. 5e), and higher content at Stages 3 and 5 in the on-plant samples (considering midrib and edge together), but no increase until Stage 5 in the

commercially treated samples. Sucrose content remained relatively constant over stages of development (considering midrib and edge together), but at each stage on-plant samples showed higher mean levels than their commercially treated counterparts with a significant difference at Stage 5 (Fig. 5f).

### 3.6. Starch content is affected by tepal sector, bud position and stem treatment

Tepal starch content was analysed in cv. 'Ascot' tepals to assess whether loss of starch might be contributing to the differential cell expansion across tepals and the changes in glucose and fructose levels related to stem treatment. Relative bud position was also considered.



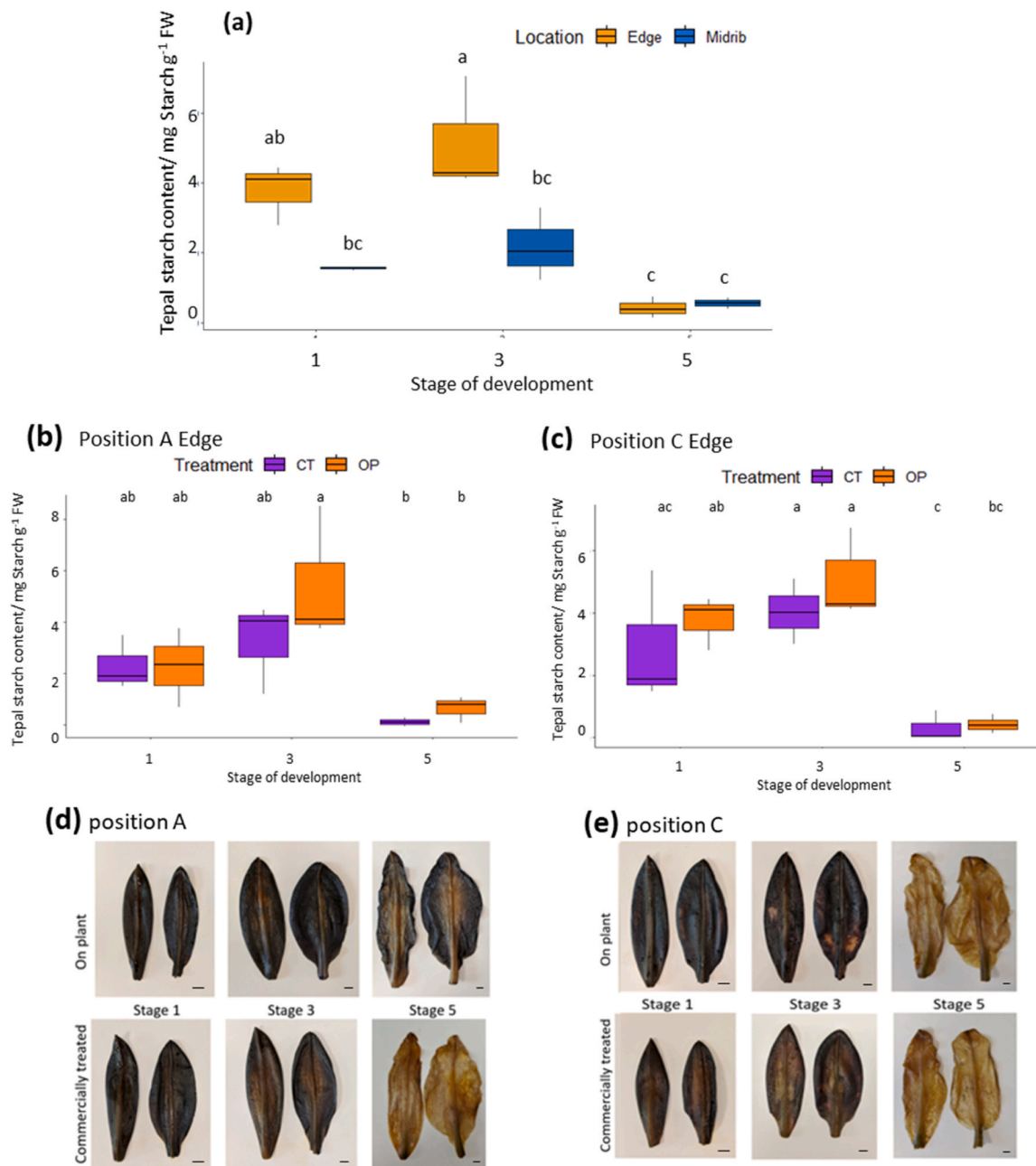
Starch on-plant remained relatively constant in both edge and midrib sectors between Stage 1 and 3 but then decreased significantly ( $P < 0.05$ ) between Stage 3 and Stage 5 in the tepal edge sector as the lily flower opened (Fig. 6a). Moreover, midrib sections had lower tepal starch content compared to edge sections at Stage 3 (Fig. 6a).

Tepal edges showed no significant differences in starch content between on-plant and commercially treated stems, either in Position A buds (Fig. 6b) or Position C buds (Fig. 6c) at any of the developmental stages. However, at Stage 5 starch content of on-plant samples of both position A and C buds fell significantly, and starch was also less in position C tepal edges of commercially treated stems compared to Stage 3. While the enzymatic starch assay showed few significant differences either regarding commercial treatment or position on stem, the Lugol stain of Oriental lily ‘Tisento’ tepals at the same stages of development

showed a visible reduction in staining in commercially treated Position A tepals compared to on-plant and at Stage 5 (Fig. 6d). Additionally, when Position A and C tepals were compared on-plant, Position C buds also showed visibly less starch staining at Stage 5 compared to Position A (Fig. 6d, e). Commercially treated position C buds also had visually less staining in Stage 1 and 3 compared to on the plant tepals, suggesting that position on stem may exert an additional effect on starch content in commercially treated buds.

### 3.7. Expression of genes related to sugar transport and cell expansion changes during bud opening

Real time qPCR was used to assess whether changes in metabolites across lily tepal development following commercial treatment could be



**Fig. 6.** Tepal starch content in Oriental lily cv. Ascot through development and flower opening comparing (a) edge and midrib tepal sections; (b) Position A edge and (c) Position C edge tepals from stems subjected to either commercial treatment (CT) or on the plant (OP) as measured by enzymatic assay. (d) and (e) comparison of starch content based on Lugol staining between on plant and commercially treated Position A (d) and Position C (e) tepals. For enzymatic assays  $n = 3$  Mean  $\pm$  SD; different letters indicate significant differences ( $P < 0.05$ ). Scale bar = 1 cm.

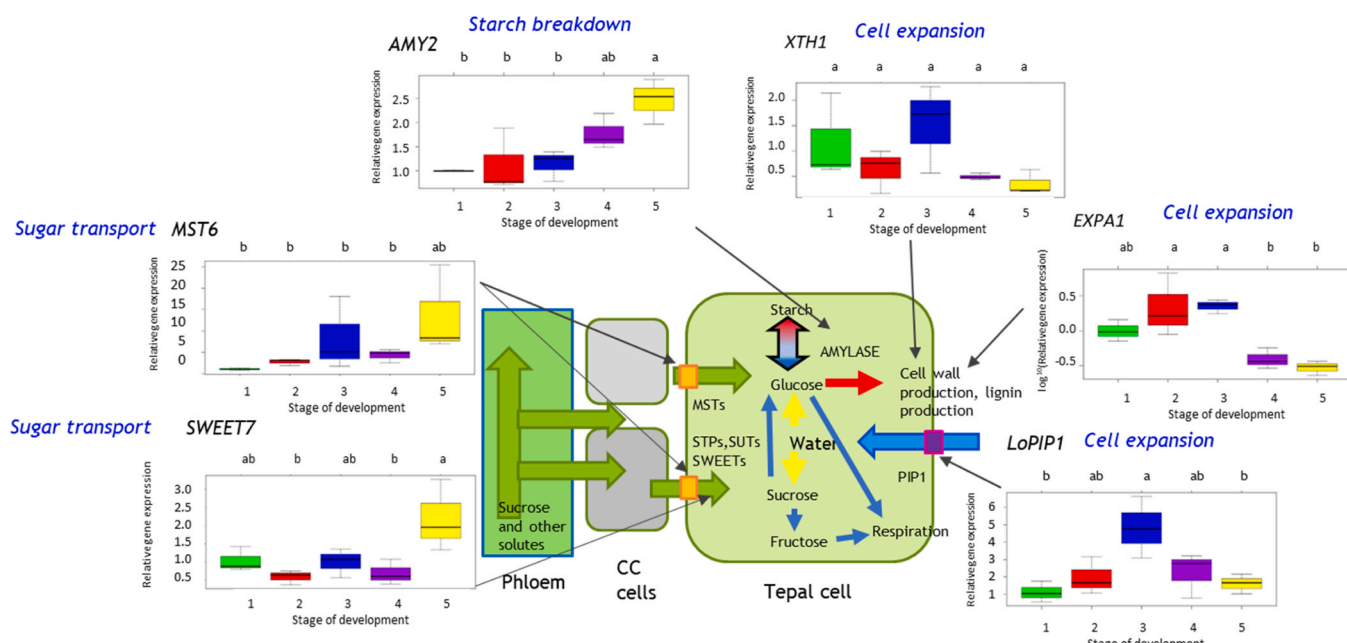
explained by changes in the expression of genes related to starch breakdown, sugar transport and cell expansion. Two genes related to sugar transport, *MST6* and *SWEET7* increased in relative gene expression over bud development with a significant increase in expression of *SWEET7* at Stage 5 compared to Stage 4 (Fig. 7). However, genes coding for other sucrose transporters, *SUT2* and *SUT4* did not show significant changes in gene expression over development and opening (Supplementary Figure 5). Relative expression of the putative *AMY2* gene coding for  $\alpha$ -amylase, involved in starch breakdown, also increased over development and opening, peaking at Stage 5 with 2.46-fold higher relative expression compared to Stage 1 (Fig. 7). Three genes relating to cell expansion showed a different pattern of expression. *EXPA1* putatively encoding an  $\alpha$ -expansin showed a significant reduction in expression in Stage 4 and 5 compared to Stages 2 and 3, and *XTH1* coding for a xyloglucan transferase-hydrolase showed a reduction in expression at Stages 4 and 5 although this was not significant, probably due to the high variability in expression at Stages 1 and 3. The putative *PIP1* gene increased significantly in relative expression from Stage 1 to Stage 3, peaking at almost 5-fold higher expression than Stage 1. At Stages 4 and 5 relative expression of *LoPIP1* decreased again to a very similar level as Stage 1. The putative gene coding for a cell wall invertase 4 (*CWINV4*) showed a possible increase over bud development Stages 1–5, but the changes were not significant due to high sample variability (Supplementary Figure 5).

#### 4. Discussion

The commercial processing of flowers involves several steps, including the cutting of stems, hydration and treatment with preservatives, controlled storage regimes, transport and dry storage. In lilies this can cause incomplete flower opening on cut stems. In order to understand the combined effect of the entire process involved in the treatment of Lilly stems, we investigated its effect, including the effect of the positions of the buds on the stems.

The cold/dark treatment (72 h, 4–5 °C) used commercially has been

reported to slow metabolism and transpiration in lilies, improving longevity (Van Doorn and Han 2011; Wei et al. 2018). This was hypothesised to slow inflorescence development, which would be consistent with the delay in bud opening observed here. However, here bud development in commercially treated stems was compared to on-plant buds; hence some of the delay may also be due to the harvesting. Sequential opening noted here is characteristic of lily flowers and is likely a method of temporally and spatially separating the pollen transfer from self- to non-self flowers (Brunet and Charlesworth, 1995). Bud opening was also sequential in commercially treated stems indicating that the endogenous signals regulating this temporal regulation remain intact postharvest and post-cold storage. However, the comparison of bud opening between on-plant and harvested/ commercially treated stems confirmed that the smallest bud frequently does not open within 7 d, a time period typically indicated by commercial retailers for freshness guarantee. Flower opening is an energetically consuming process for the plant (van Doorn and Kamdee, 2014) and successive buds do not develop independently from each other. In lilies post-harvest experiments indicate translocation of nutrients from older buds/open flowers to young developing buds (van der Meulen-Muisers et al., 2001) indicating that young buds are a strong nutrient sink. Indeed, addition of sugars to vase water stimulated bud opening (e.g. Arrom and Munné-Bosch, 2012). The expression of two genes related to phloem loading and unloading: *MST6* and *SWEET7* was assessed over bud development but they were both only upregulated late in flower opening, and indeed the rise in *MST6* was not statistically significant. This does not exclude a role for them earlier in bud development, but the late up-regulation could be linked to phloem loading out from the flower to other developing buds once the flowers have outlived their reproductive potential (Van Meeteren et al., 2001). Other genes involved in phloem loading/unloading: *SUT2*, *SUT4* and *CWINV4* were expressed at a constant level during bud development and flower opening suggesting that in terms of sugar transport into the bud, transcriptional regulation may not play an important role. Indeed, Xu et al. (2018) suggested that although phloem loading is highly responsive to external stimuli,



**Fig. 7.** Expression of genes relating to phloem loading strategies (symplastic and apoplastic). Symplastic strategies (outlined in blue) show the movement of sucrose from the phloem into companion cells (CC) and then into tepal cells via sucrose transporter proteins (STPs/ SUTs) and Sugars Will Eventually be Exported transporters (SWEETs), entering the cell as sucrose. Monosaccharides produced can cause cell expansion by increasing osmotic pressure in the cell or be used in lignin and cell wall production. Diagram based on Ruan et al. (2010), Lemoine et al. (2013), Ruan (2014), Durand et al. (2018). qPCR values represent medians of 3 biological replicates  $\pm$  SD. Different letters indicate significant differences based on a one-way ANOVA with post-hoc Tukey or Kruskal-Wallis test with post-hoc Dunn ( $P < 0.05$ ).

up-regulation is more likely to be caused by post-transcriptional processes.

The cellular and global metabolic changes during opening required further investigation given that they may contribute to the failure of young buds to open post-harvest. Although cell diameters (Bielecki et al., 2000) and areas (Watanabe et al., 2022) had been measured between adaxial and abaxial surfaces of lily tepals, questions remained on the contribution of the other two dimensions: cell expansion proximal and distal to the midrib and at the tip compared to the base of the tepal. The more rapid expansion of adaxial cells noted previously (Bielecki et al., 2000) was confirmed here; however, the expansion in edge and midrib appeared to proceed at a very similar rate, albeit with larger edge cells throughout both in buds following commercial treatment and on plant. The significant variation in the rate of pavement cell area change across the tepal sectors supports the hypothesis that differential growth is required for flower opening in lilies (Bielecki et al. 2000; Liang and Mahadevan, 2011; Watanabe et al. 2022). Moreover, there appears to be a rapid increase in cell area in the middle sector of the tepal, most pronounced in the adaxial surface which may be critical in the inflexion change from convex to concave. The expression of *PIP1* and *EXPA1* is in agreement with previous studies (Watanabe et al., 2022) showing that they are transcriptionally upregulated just prior to flower opening and therefore may be important in the opening process. The relatively constant expression of *XTH1* during bud expansion and flower opening may indicate transcriptional regulation of this gene is not relevant to flower opening (Watanabe et al. 2022), although there is a slight increase at Stage 3 of bud development, masked by the variability across the buds.

It was hypothesised that cell expansion is driven in lily buds through changes in osmotic pressure, driven in turn by changes in soluble sugar content, related to starch breakdown, sucrose uptake from phloem unloading, or both (Bielecki et al. 2000, Van Meeteren 2001). Starch breakdown was previously shown to be important for tepal expansion (Bielecki et al. 2000). Mean starch content in Oriental lily ‘Sorbonne’ tepals on-plant was also found to peak at mature bud stage at around  $3.5 \text{ mg g}^{-1} \text{ FW}$  and then drop to less than  $0.5 \text{ mg g}^{-1} \text{ FW}$  in the fully open flower (Watanabe et al. 2022), and to under  $3 \text{ mg g}^{-1} \text{ FW}$  in commercially treated Asiatic hybrid lily ‘Enchantment’ tepals (Clément et al. 1996). Data here showing a loss of starch over bud development and flower opening supports these earlier studies, as does the continuous upregulation of the *AMY2* gene. Moreover, this study showed that tepal edge sections had significantly higher starch content in Stages 1 and 3 compared to midrib sections. This suggests that tepal edges may retain a greater starch store and higher metabolism during flower opening. This would also be consistent with the smaller size of edge cells. However, the greater expansion of middle/edge adaxial cells during bud opening is not supported by a faster starch breakdown indicating that other mechanisms may be involved. Data presented here also show that the fall in starch mirrored an increase in glucose and fructose over flower opening as has been found previously (Watanabe et al., 2022). The tepal glucose, fructose and sucrose content was measured here as the major soluble sugars in lily flowers (Clément et al. 1996; Watanabe et al. 2022). Higher concentrations of soluble sugars (glucose, sucrose, fructose) in tepal edge sections compared to tepal midrib sections are not reflected in greater starch breakdown and may indicate that sugars are being imported into this sector of the tepal to drive expansion.

The reduced content of glucose, fructose and sucrose, in open flowers subjected to commercial treatment compared to on-plant is consistent with a nutritional deficiency and in line with studies showing that sugar supplementation can stimulate bud opening (Arrom and Munné-Bosch, 2012), although it is also possible that starch breakdown, perhaps through changes in the expression of starch breakdown-related genes, is affected by cutting the stems. Here sucrose levels remained relatively constant through bud development and flower opening, contrasting with previous data (Watanabe et al., 2022) and may reflect varietal differences. The increase in flower opening and acceleration in opening speed in cut lily flowers when sucrose was added to the vase solution

(Han, 2003) suggested that the starch content in tepals may be insufficient and that a higher tepal soluble sugar content is required for opening.

The analysis of the whole metabolome indicated global differences in metabolites between the edge and midrib of the tepals. This is consistent with differences in sugar content seen between adaxial and abaxial tepal cells (Watanabe et al., 2022) and is further evidence of localised changes which may be relevant to the mechanism of flower opening. Metabolomic profiling also suggested that commercial treatment has a significant impact on the metabolome of flowers regardless of stage of development. This is again consistent with a nutritional and metabolic differences elicited by harvest and the cold/dark treatment. Although a small minority of the compounds could be identified, the finding that several secondary metabolites are upregulated by the commercial treatment throughout bud development may indicate the activation of stress responses as has been found in other flowers (Wagstaff et al. 2010). The down-regulation of a monoterpenoid, on the other hand may be due to a repression of scent production in the cold, also reported in other flowers (Sagae et al. 2008). There was no clear pattern of changes in specific metabolite families between edge and midrib, however differences in phenolics and flavones/flavonoids are most likely to be related to scent and pigment production rather than flower opening as these are likely to vary across the tepal (Yin et al. 2020). The main metabolites that differed in abundance related to bud position were phospholipids. Phospholipids are the major component of cell membranes and alongside structural roles, some are essential as secondary messengers to regulate growth, development, senescence, and stress responses (Xue et al. 2007). Phospholipid and fatty acid content are also important in thermotolerance (Liang et al., 2023). This may indicate differences in tolerance between different bud positions. It may also reflect differences in resource allocation resulting in differential biosynthetic programmes.

## 5. Conclusions

The data shown here confirm that the physical bud opening process is linked to localised differential epidermal pavement cell expansion across the tepal, with complex interactions amongst tepal areas. This cell expansion may be driven both by starch breakdown and sugar uptake. The commercial processing of lily stems, encompassing harvest, treatments, storage and transport, reduced tepal sucrose and glucose levels but not starch content. This suggests that the uptake of soluble sugars via the phloem may be compromised in cut cold/dark stored stems. Commercial treatment delayed bud opening but did not have a major effect on cell expansion rates. Differences in tepal starch content of buds at different positions on stems suggests potential targets for breeding to maximise starch and soluble sugar maintenance in tepals postharvest thus enabling all buds on the stem to open.

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## CRedit authorship contribution statement

**Walter Dewitte:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Manfred Beckmann:** Methodology, Investigation. **Rakhee Dhorajiwala:** Writing – original draft, Investigation. **Hilary J Rogers:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Paul F. Devlin:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Anthony D. Stead:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hilary J Rogers reports financial support and equipment, drugs, or supplies were provided by Chrysal International BV. Hilary J Rogers reports a relationship with Chrysal International BV that includes: funding grants and travel reimbursement. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.postharvbio.2024.113063](https://doi.org/10.1016/j.postharvbio.2024.113063).

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