Volatilome and aroma appreciation of ready to eat strawberry fruit with altered *alcohol acyltransferase* gene expression during postharvest storage.

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Abstract

Strawberries are a desirable component of fresh fruit salads. They are known to be highly perishable and are therefore stored at low temperature to prolong their shelf-life. Chilled storage, however, impacts biochemical and physiological processes in the fruit, affecting consumer appreciation and causing food waste. Aroma is an essential characteristic of strawberries and is known to change due to chilled storage. Aroma profiles also differ across different strawberry varieties. We assessed changes in volatile organic compound (VOC) profiles of halved and cold stored fruit of three *Fragraria x ananassa* cultivars: 'Elsanta', 'Favori', and 'Malling Centenary'. 'Favori' produced the most complex aroma with a total of 132 VOCs identified. The 'Favori' VOC profile was also least affected by storage, while the most affected was 'Elsanta'. A recent transcriptomic study showed that chilled storage changed expression of a large number of genes. We developed a transient gene expression method for post-harvest strawberries to overexpress genes relevant to VOC biosynthesis, aimed at understanding better the molecular mechanisms behind VOC changes during chilled storage. We tested the method by transiently transforming three different cultivars ~~storage~~ with the fluorescent protein marker mCherry. After demonstrating that the marker was successfully expressed even after several days of storage, we then tested the effects of expression of *alcohol acyltransferase* (*AAT*). The gene is involved in ester production, an important VOC family in strawberry aroma and over-expression of *AAT* was assessed after post-harvest storage by recording VOC profiles of the fruits. We show that over-expression of *AAT* can modulate VOC profiles after storage and that this can be perceived by consumers. We anticipate that our approach will help to identify useful markers for breeding strawberry fruit with better retention of aroma during postharvest storage, of clear benefit to the fresh-cut salad industry.

Keywords: *Fragraria x ananassa* gene expression, alcohol acyltransferase, transient transformation, postharvest storage.

INTRODUCTION

Strawberries (*Fragaria x ananassa*) are a non-climacteric fruit, thus ripening needs to occur on the plant, and strawberry fruit are picked at full maturity when they are in their final stages of ripening. Partly as a result of this, they are highly perishable as they are less firm at harvest than climacteric fruit, increasing their susceptibility to mechanical damage and spoilage microorganisms (Siebeneichler et al. 2020). To limit deterioration fruit are stored and transported at low temperatures to slow down

ripening and to limit the growth of spoilage microorganisms (Cordenunsi et al. 2003). Although low temperature slows general metabolism and activities of enzymes in the fruit and therefore reduces the rate of structural changes, it also upregulates stress responsive genes and affects both primary and secondary metabolism (Brizzolara et al. 2020). Therefore, this negatively impacts many processes associated with quality traits including colour, texture, taste, aroma and nutrition (Pott et al. 2020).

Strawberries are a valued component of ready to eat (RTE) fresh fruit salads, used either whole or halved. However, processing for RTE shortens the shelf-life of the fruit (Ma et al. 2017). The processing ~~packaging~~ elicits physiological changes associated with wounding stress which includes increased respiration rates as well as an increase in the production of secondary metabolites such as phenolics and off-flavours, cell wall degradation and softening (Baldwin and Bai 2010).

Strawberry fruits are judged by consumers based on many factors including size, shape, colour, firmness, aroma, acidity, sweetness, and overall fruit flavour (Roussos et al. 2009). Aroma is a key component of strawberry fruit appreciation by consumers because it plays a key role in determining fruit flavour (Ahmed et al. 2013). The overall fruit aroma results from a complex mixture of numerous volatile organic compounds (VOCs) which is usually species-, variety- and organ-specific (Schwab et al. 2008). *Fragaria x ananassa* has a very complex aroma, made up of over 350 volatile organic compounds (VOCs) and maybe up to >900 (Ulrich et al., 2018; Rey-Serra et al. 2022). Esters comprise 90% of the total number of different VOCs (Jetti et al. 2007). However, of all the strawberry VOCs, only a subset are considered important for the characteristic strawberry aroma (Urrutia et al. 2017): methyl butanoate, ethyl butanoate, methyl hexanoate, hexyl acetate, ethyl hexanoate, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (HDMF, furaneol) and its methyl ether (Larsen and Watkins 1995; Jetti et al. 2007). The combined contribution of these odour-active compounds result in the characteristic fruity, sweet, green and floral aroma of strawberry fruit.

Aroma changes during storage of fruit including strawberries (Muto et al., 2022; Spadafora et al., 2015; Baldwin et al., 2023; Li et al. 2021; Ayala-Zavala et al. 2004). The alcohol acyltransferase (AAT) enzyme catalyzes the last stage of ester synthesis: the esterification of acyl groups from acyl-CoA onto alcohols (Navarro-Retamal et al. 2016). During cold storage, in both peach and tomato fruit, ester abundance and *AAT* gene expression decreased (Zhang et al. 2011; Zhang et al. 2016).

Here we explored the effects of storage on the whole aroma profile of different strawberry varieties and assessed the effect of transiently over-expressing the *AAT* gene on VOC profile and sensory perception. Our hypothesis was that increasing *AAT* expression might reduce aroma loss during storage and might therefore provide a useful breeding target to improve post-harvest aroma retention.

MATERIALS AND METHODS

Plant material and treatments

Three cultivars of strawberry (*Fragaria x ananassa*) fruit were used: cv. Favori were grown at an indoor farm (Wales UK), while cv. Malling Centenary and Elsanta were grown outdoors (Wales UK). All fruit were handpicked and processed within 3 hours. Fruit were processed as previously described (Baldwin et al., 2023) and halved fruit were stored for 5 days at 8 °C.

Analysis of volatile organic compounds

VOCs were collected from the headspace of strawberry fruit held at room temperature for 2 h onto thermal desorption tubes with three biological replicates, and subjected to thermal desorption gas chromatography time of flight mass spectrometry also as previously described (Baldwin et al., 2023). Statistical analysis was performed as

described previously (Baldwin et al., 2023) using canonical analysis of principal components (CAP).

Transient transformation of strawberry fruit

The binary vector pL2B-Kan-pLjUBI-GUS-tNOS-p35S-mCherry-t35S for testing the transformation system was kindly provided by National Institute of Agricultural Botany (NIAB). The *AAT* coding region was synthesized based on the genome sequence (gene33976) and cloned using the Golden Gate cloning system and a MoClo Plant Parts Kit (Engler et al. 2014) according to kit instructions. The coding region was designed with BbSI restriction enzyme overhangs and cloned into the vector BsaI restriction enzyme site. Plasmids were transformed into *Agrobacterium tumefaciens* EHA105 and 1 mL of an overnight culture was pelleted at 8000 x g for 2 minutes and resuspended in 1 mL of infection buffer (10 mM $MgCl_2$, 100 μM acetosyringone, and 10 mM MES, pH 5.6). The resuspended bacteria were injected into strawberry fruit essentially as described in Zhao et al. (2019). mCherry expression was detected using a Biospace Lab PhotonIMAGER

Sensorial analysis of strawberry fruit

Participants , recruited on the day in the main foyer of the School of Biosciences, Cardiff University were asked to smell strawberry fruit in sealed boxes through a grating in the lid. Fruit comprised those injected with *Agrobacterium* carrying an empty vector, the vector with the *AAT* construct and uninjected fruit controls. Fruit were stored at 8 °C for 5 days prior to the sensorial test. Participants were asked to smell samples and rate them on a Likert scale from 1-9 with 1 being extremely disliked and 9 being extremely liked, as well as ranking the samples by their order of preference as first to third. The samples were coded and the order of the samples to be assessed by participants was randomised on each questionnaire to avoid any order bias. Two sensorial experiments were conducted, one with 'Elsanta' fruit and the other with 'Malling Centenary' fruit. Ethical approval was obtained from the School of Biosciences Research Ethics Committee, (Approval no. SREC 22 06-02) and the analyses were in compliance with local rules and UK legislation on handling of genetically modified organisms under contained use. Statistical analysis was carried out in R studio (Version 1.4.1103, R version 4.1.1) using a Kruskal–Wallis test followed by the Dunn post-hoc test. Data for the two varieties were combined for extra statistical power (n=150).

RESULTS

1. Aroma change in different strawberry varieties during postharvest storage.

Across the cultivars there were 110 VOCs detected in cv. Elsanta, 132 in cv. Favori and 99 in cv. Malling Centenary. 44% of the VOCs were found in all three cultivars. Changes in aroma profiles in response to chilled storage were assessed on halved fruit of the three cultivars: Elsanta, Malling Centenary and Favori (Fig 1).

Linear discriminant plots of whole VOC profiles from CAP show that in both cv.s Elsanta and Malling Centenary there is complete discrimination between fruit before and after storage. On the contrary, the VOC profiles before and after storage of cv. Favori fruit were not discriminated. The percentage of correct classifications for 'Elsanta', 'Malling Centenary' and 'Favori' was 83.3%, 83.3% and 72.2% respectively.

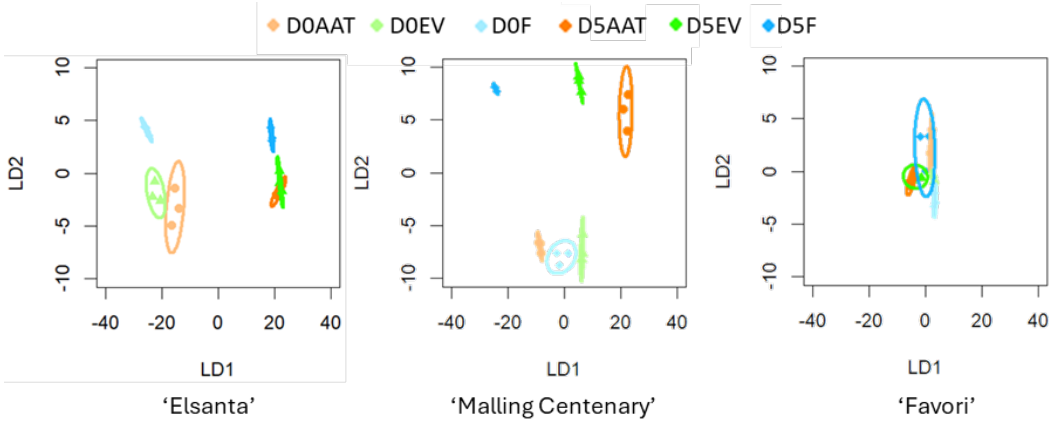

Figure 1: Linear discriminant plots of CAP of VOC profiles for three strawberry cultivars at day 0 (D) and day 5 (D5) of chilled storage. Fruit were either untreated (F) or Agroinfiltrated with the construct carrying the *AAT* gene (AAT) or empty vector (EV). Ellipses show 95% confidence intervals (SD).

2. Transient transformation of strawberry fruit and the effect on the aroma profile.

To test whether it was possible to detect expression of a transgene during post-harvest storage following Agroinfiltration of strawberry fruit, fruit were injected with *Agrobacterium* carrying an mCherry fluorescent protein coding region driven by a strong constitutive promoter. After 5 days of storage at 8 °C the mCherry signal was still very clear (Fig. 2).

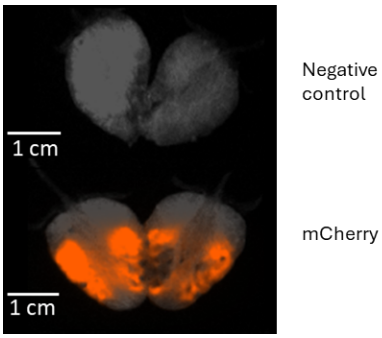

Figure 2. cv. Elsanta strawberries Agroinfiltrated 3 days on plant before harvest then stored for 5 days at 8 °C. The fruit was injected at turning stage. Scale is in cm. Orange patches show the expression of the mCherry fluorescent protein.

Whole VOC profiles were compared using CAP and linear discriminant plots across the three treatments: untreated fruit, empty vector and vector carrying the AAT gene (Fig. 1). In 'Malling Centenary' there was clear separation of VOC profiles between the three treatments after 5 days of storage (D5AAT, D5EV and D5F), while in cv. Elsanta there was only discrimination between not injected and injected fruit. In cv. Favori, there was no clear discrimination amongst the three VOC profiles.

3. Sensorial analysis

Sensorial analysis was used to test whether expression of the *AAT* construct affected the sensorial perception of fruit aroma. in 'Elsanta' fruit and 'Malling Centenary' fruit. Overall, the 150 respondents showed a preference for the fruit carrying the *AAT*

construct compared to the empty vector or uninjected fruit, although when asked to assign a Likert score there was no significant difference in their response between the AAT and empty vector injected fruit (Fig. 3)

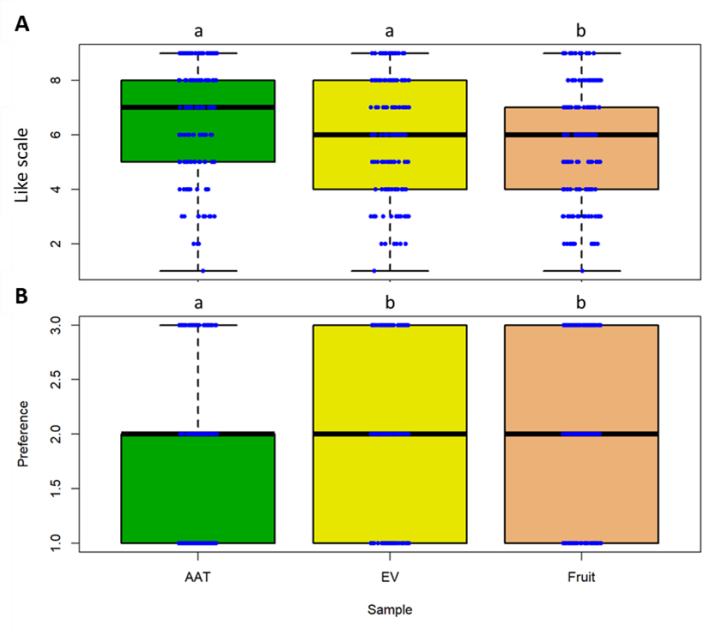

Figure 3: Sensorial analysis of the aroma from strawberry fruit (cv.s Elsanta' and 'Malling Centenary') injected with *Agrobacterium* carrying different constructs (the *AAT* construct (AAT) or the empty vector (EV)) or not injected fruit control. A) scores on a 1-9 Likert scale where 9 is most liked; B) ranked preference 1-3 where 1 is most preferred and 3 is least preferred. Responses with different letters indicate statistically significant differences (Kruskal-Wallis, $P<0.05$, $n=150$).

DISCUSSION

The number of VOCs detected in the strawberry samples is consistent with previous studies (Ulrich et al., 2018). Although over 900 VOCs have been reported across the many studies on strawberry aroma, many of them were only reported once and it is likely that composition may vary with variety as well as detection limits varying across different analysis platforms. The effect of chilled storage on strawberry aroma is also well-known (e.g. Baldwin et al., 2023; Li et al. 2021; Ayala-Zavala et al. 2004) and was confirmed here in both 'Elsanta' and 'Malling Centenary'. Surprisingly the aroma of cv. Favori remained relatively unchanged by storage with the day 0 and day 5 of storage patterns overlapping at the 95% confidence interval. This may indicate that this variety is particularly good at retaining flavour through storage.

The mCherry fluorescence indicated successful Agroinfiltration of the fruit and transient expression of the transgene. This is similar to previous results by Miyawaki et al. (2012) who also using detached fruit found that GFP fluorescence expression was observed until 5 days after infiltration. This was important for this study as it demonstrated that the Agroinfiltration method could be used to study gene overexpression during post-harvest.

Since previous studies had shown that esters were a key component of the aroma (Jetti et al., 2007) alcohol acyltransferase (AAT) was selected as it catalyzes the last stage of ester biosynthesis. Our hypothesis was that 5 days of storage was sufficient

time for over-expression of *AAT* gene in the strawberry fruit to be translated, and the enzyme activity to affect aroma composition. Analysis of the aroma profiles show that there are significant differences in VOC profile between untreated fruit and the agroinfiltrated fruit after 5 days of storage in two of the cultivars tested, and indeed that differences between the three treatments in 'Malling Centenary' were more pronounced after the 5 days of storage as might be expected. The lack of change in profiles following Agroinfiltration in cv. Favori is consistent with its lack of change in storage and may indicate perhaps that there is insufficient substrate for the AAT to act on. The lack of discrimination between empty vector and *AAT* agroinfiltrated cv. Elsanta' fruit may indicate an effect of the infiltration process which may induce a defence response eliciting the production of VOCs (Guidarelli and Baraldi 2015).

Sensorial analysis indicated that respondents were able to discriminate the three fruit treatments by their aroma. Discrepancy between the Likert scale and preference results probably reflect the subtle differences between the aromas. However, the sensorial analyses indicate that the Agroinfiltration of the *AAT* construct may affect aroma. Further work is required to assess if the ester profile has been changed which would relate more specifically to an effect of AAT enzyme activity and might indicate that this might be a useful breeding target for aroma retention during shelf-life.

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