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# Characterisation of two alcohol acyltransferases from kiwifruit (*Actinidia* spp.) reveals distinct substrate preferences

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ABSTRACT

Volatile esters are key compounds of kiwifruit flavour and are formed by alcohol acyltransferases that belong to the BAHD acyltransferase superfamily. Quantitative RT-PCR was used to screen kiwifruit-derived expressed sequence tags with proposed acyltransferase function in order to select ripening-specific sequences and test their involvement in alcohol acylation. The screening criterion was for at least 10-fold increased transcript accumulation in ripe compared with unripe kiwifruit and in response to ethylene. Recombinant expression in yeast revealed alcohol acyltransferase activity for *Actinidia*-derived *AT1*, *AT16* and the phylogenetically distinct *AT9*, using various alcohol and acyl-CoA substrates. Functional characterisation of AT16 and AT9 demonstrated striking differences in their substrate preferences and apparent catalytic efficiencies ($V'_{max}$ $K_m^{-1}$). Thus revealing benzoyl-CoA:alcohol *O*-acyltransferase activity for AT16 and acetyl-CoA:alcohol *O*-acyltransferase activity for AT9. Both kiwifruit-derived enzymes displayed higher reaction rates with butanol compared with ethanol, even though ethanol is the main alcohol in ripe fruit. Since ethyl acetate and ethyl benzoate are major esters in ripe kiwifruit, we suggest that fruit characteristic volatile profiles result from a combination of substrate availability and specificity of individual alcohol acyltransferases.



## 1. Introduction

Until thirty years ago plant secondary metabolites were mainly recognised as "metabolic waste products" (Peach, 1950). However this perspective has changed remarkably with more than 200,000 different compounds identified to date. These phytochemicals perform a variety of roles in plants from defence against abiotic and biotic stresses through to attraction and stimulation of its biological environment (Hartmann, 2007). A large number of these molecules are produced by acylation of a hydroxyl, amino or thiol-group, catalysed by acyltransferases (EC 2.3.1.x). Of particular importance are members of the BAHD (**b**enzyl alcohol-acetyl-, **a**nthocyanin-*O*-hydroxy-cinnamoyl-, anthranilate-N-**h**ydroxy-cinnamoyl/benzoyl-, **d**eacetyl-vindoline) acyltransferase (AT) superfamily that produce a wide range of functionally important compounds such as lignin, phenolics, alkaloids, phytoalexins, anthocyanins and volatile esters (St-Pierre and De Luca, 2000; D'Auria, 2006). BAHD ATs are generally recognised by their active site motif (HXXXD) and a conserved region (DFGWG) with likely structural significance (Ma et al., 2005). Like most ATs, BAHD proteins use Coenzyme A (CoA)-thioesters as acyl donors and one subgroup of BAHD ATs specifically forms esters by aliphatic or aromatic *O*-acylation of alcohol acceptor molecules.

Volatile esters produced by these alcohol acyltransferases (AATs) often drive plant-food recognition because they contribute to the "fruity" aroma of edible fruits. Some esters are also responsible for specific, key flavours or odours (Morton and Macleod, 1990). In kiwifruit, for example, elevated levels of methyl and ethyl butyrate have been recognised as characteristic fruit aroma compounds (Gilbert et al., 1996). Furthermore, these compounds dominate (Fig. 1) the fruit ester profiles of the commercial kiwifruit cultivars *Actinidia deliciosa* var. deliciosa 'Hayward' and *Actinidia* Planch. var. *chinensis* 'Hort16A' and reach peak levels at the soft end of their eating firmness range (Wang et al., 2011). Additional alkyl substituted esters, including (methylsulfanyl)alkanoate and benzoate esters, have been identified from the volatile profiles of 'Hayward' and 'Hort16A' kiwifruit (Young and Paterson, 1985; Friel et al., 2007; Günther et al., 2010; Wang et al., 2011). The major alcohol detected in fruit of these cultivars was ethanol (Fig. 1) with at least 50% of the resulting volatile esters being ethyl esters.

Although changes in kiwifruit ester profiles have been well researched in response to ethylene, harvest maturity and postharvest treatment (Young and Paterson, 1985; Günther et al., 2010, 2011; Wang et al., 2011), the genes involved in ester biosynthesis remain to be elucidated. As a result of an expressed sequence tag (EST)

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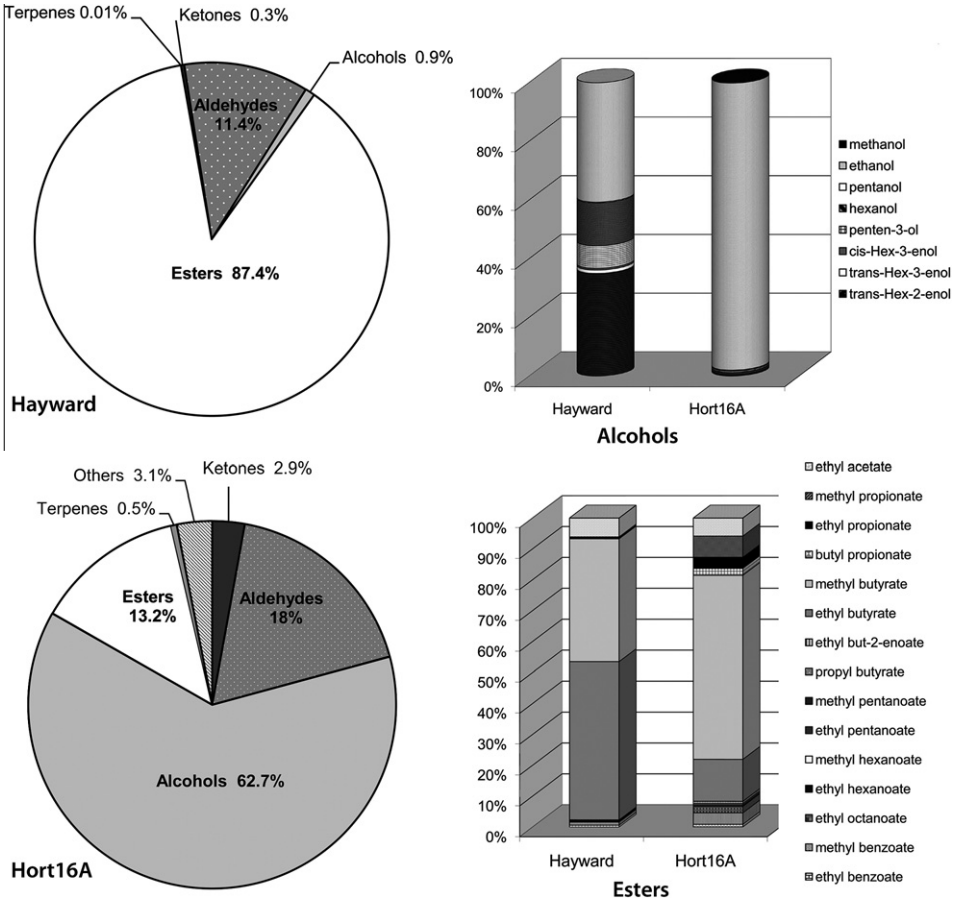


**Fig. 1.** Kiwifruit volatiles as% of total volatiles, alcohols as% of total alcohols, and esters as% of total esters. Volatile data for *Actinidia deliciosa* 'Hayward' were taken from "overripe" fruit (Young and Paterson, 1985). *Actinidia chinensis* 'Hort16A' volatile levels correspond to "soft" fruit as reported by (Friel et al., 2007). Alcohol and ester compounds are displayed in the same order as listed in keys to the right of the figure.

sequencing project from mainly four different *Actinidia* species (*A. deliciosa*, *A. chinensis*, *Actinidia arguta*, *Actinidia eriantha*) 25 full-length clones with putative AT and AAT functions have been identified (Crowhurst et al., 2008). Twelve of these contig-sequences were suggested to be flavour-related due to their phylogenetic relationship to characterised AATs from melon (CmAAT1, El-Sharkawy et al., 2005), apple (MpAAT1, Souleyre et al., 2005), banana (BanAAT, Beekwilder et al., 2004) and strawberry (SAAT and VAAT, Beekwilder et al., 2004).

Here, we investigated changes in steady-state transcript levels of *Actinidia* ATs to select sequences, potentially encoding for AATs that may be involved in ester biosynthesis, specific for ripe kiwifruit. We then studied the substrate preferences of these encoded enzymes using recombinant expression in order to evaluate their potential role for flavour-related fruit ester formation. Finally, the results are discussed in the light of their phylogenetic relationship with fruit and flower-derived AATs from other species.

## 2. Results and discussion

### 2.1. Isolation of putative ATs that are ripening-related using qRT-PCR analysis

Our previous studies (Günther et al., 2010, 2011) implied that enhanced volatile ester production during kiwifruit ripening was linked to ethylene-induced expression of *AATs*. Therefore, qRT-PCR analysis of *Actinidia* EST-contigs (Crowhurst et al., 2008) (all full-length-clones except for *AT15*) with putative AAT and AT-function was performed using RNA isolated from *A. deliciosa* 'Hayward' and *A. chinensis* 'Hort16A' that were softened to eating ripeness either with or without ethylene treatment as described in Nieuwenhuizen et al. (2007).

Ratios of the relative amounts of transcripts from ripe versus unripe fruit are displayed in Fig. 2. In *A. chinensis* 'Hort16A' (Fig. 2B) transcript levels of six contig sequences (*AT18*, *AT2*, *AT15*, *AT1*, *AT17*, *AT16*; isolated from *A. deliciosa*, *A. chinensis*, *A. arguta*) were 10 (*AT18*) to 550 (*AT15*) fold increased in ripe compared with unripe fruit, and 20 (*AT16*) to 2000 (*AT15*) fold after ethylene treatment. Except for *AT16*, transcript levels of these same contigs also differed from the majority of *ATs* in *A. deliciosa* 'Hayward' (Fig. 2A) showing 30 (*AT1*) to 100 (*AT18*) fold increased accumulation in ripe ethylene treated and untreated versus unripe fruit. Interestingly, transcript levels of *AT16*, which was isolated from *A. chinensis*, were only two-fold increased in ripe compared with unripe *A. deliciosa* 'Hayward' fruit and even two-fold decreased after ethylene-treatment. It has been suggested that *AT1*, *AT17* and *AT16* (Crowhurst et al., 2008) are orthologues, and it appears likely that ethylene regulation of *AT16* diverged upon speciation. Transcripts of *AT10* and *AT12*, isolated from *A. eriantha* and *A. deliciosa*, accumulated approximately five-fold in ripe 'Hort16A' but 15- and 40-fold in ethylene-treated 'Hayward' fruit, respectively. This suggests that the steady-state transcript levels of these sequences are likely to be ripening-related in *A. deliciosa*. Interestingly, transcript accumulation of *AT14*, *AT7*, *AT8* and *AT23* (isolated from *A. deliciosa* and *A. chinensis*) were lower or absent in 'Hayward' and 'Hort16A' fruit after ethylene treatment compared with unripe fruit. This suggests that this ripening hormone suppressed the expression of the corresponding genes, which