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Characterisation of Thai strawberry (*Fragaria* × *ananassa* Duch.) cultivars with RAPD markers and metabolite profiling techniques

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Abstract

Strawberries (*Fragaria* × *ananassa* Duch.) are one of the most economically important fruit crops worldwide, several commercially viable cultivars are cultivated in the northern region of Thailand. The morphological characters at the young vegetative seedling stage can be very similar, which has hindered breeding efforts. The present study assesses the ability of random amplification of polymorphic DNA (RAPD) markers and metabolomics techniques to distinguish six strawberry cultivars. Both techniques showed congruent results for the leaf tissue and classified the cultivars into three major clusters. For the most different cultivars, Akihime and Praratchatan No.80, fruits were analysed at eight fruit ripening stages. The data

highlighted a broad biological variation at the early ripening stages and less biological variation at the mature stages. Key metabolic differences included the polyphenol profile in Praratchatan No.80 and fatty acid synthesis/oxidation in Akihime. In summary, the RAPD and metabolite data can be used to distinguish strawberry cultivars and elucidate the metabolite composition of each phenotype. This approach to the characterisation of genotypes will benefit future breeding programmes.

1. Introduction

The cultivated strawberry (*Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier) is a natural hybrid of *Fragaria chiloensis* (L.) Mill. and *Fragaria virginiana* Mill. both part of the *Rosaceae* family (Ara et al., 2013; Sakila et al., 2007). The octoploid (2n = 56) herb with perennial and stoloniferous properties is grown around the world including Thailand (Darrow, 1966; Karim et al., 2011). The northern provinces of Thailand (Chiang Mai and Chiang Rai) are especially suitable for cultivating strawberries due to the cooler weather (Sirijan et al., 2019). The breeding programme in northern Thailand resulted in six strawberry cultivars highly desired by consumers. These cultivars include Praratchatan No.50, Praratchatan No.70, Praratchatan No.72, Praratchatan No.80, No.329 and Akihime and have been tested for good aroma, redness, sweetness, firmness and high antioxidant (Pipattanawong et al., 2011). Currently, Thailand has no procedures in place to certify and assess true-to-type cultivars for breeding programmes and producers. These procedures are necessary as it is very difficult to distinguish strawberry plants at the vegetative stage.

A molecular approach is preferred for certification as morphological characteristics can be influenced by environmental conditions (Degani et al., 1998; García et al., 2002). One of the widely used genotyping techniques being random amplified polymorphic DNA (RAPD) (Kuras et al., 2004; Morales et al., 2011; Zebrowska and Tyrka, 2003). This technique uses random primer binding to whole genomic DNA, which can be performed at any stage of the plant development and without previous information about the genome. Moreover, the technique is fast, easy and inexpensive and the decamer primers bind to a sufficient number of polymorphic genetic regions for agricultural purposes (Congiu et al., 2000; Gaafar and M.M, 2006). Whole genome information has recently been published for cultivated strawberry, updating the information already available for woodland strawberry (Edger et al., 2019; Shulaev et al., 2011). Hence, chemotyping of cultivars is necessary to establish the metabolic composition underlying the phenotype of each cultivar (Schauer and Fernie, 2006). Previous studies established metabolomics techniques to evaluate nutritional content beneficial for

human health, volatile profiles of strawberry aroma and investigate metabolic changes throughout fruit development (Aprea et al., 2009; D'Urso et al., 2018; de Boishebert et al., 2006; Lopes-da-Silva et al., 2002; Zhang et al., 2011).

The present study applied the published techniques to the six favoured Thai strawberry cultivars for characterisation. RAPD and metabolic analysis was performed on leaf tissue to assess similarities between the cultivars and the different levels of cellular regulation (genome and metabolome). The comparison showed very close relation between the polymorphic regions detected and the primary leaf metabolism. The six cultivars showed clear separation and two cultivars, Praratchatan No.80 and Akihime, were chosen for a more detailed metabolite profiling of the fruit over ripening. This profiling included primary and specialised metabolism as well as the volatile aroma composition. The data highlighted key differences in the metabolic regulation of the two cultivars.

2. Results and Discussion

2.1 RAPD analysis of six strawberry cultivars

RAPD markers were used to identify and assess the genetic diversity of six strawberry cultivars from Thailand. The results showed a variety of RAPD profiles for 25 decamer primers which in turn showed distinct DNA banding patterns for the six strawberry cultivars (Supplementary Table 1). The number of bands ranged from 16 bands for primer OPB05 to five bands for OPG02. These results are consistent with previous publications which showed that both primers can be used to distinguish strawberry cultivars despite the different number of distinct bands (Gidoni et al., 1994; Sugimoto et al., 2005). Overall, 263 bands were detected for 25 primers of which 87.9% were polymorphic and 13.1% were monomorphic. The PCR products were between 200-5000bp, 19 primers showed cultivar specific PCR bands and 14 primers generated 100% polymorphism (Supplementary Table 1 and 2). Praratchatan No.50 and No.80 had the highest number of cultivar specific PCR bands (thirteen and eight, respectively). The other cultivars had four or less cultivar specific PCR bands. Based on previous publications, this RAPD data is sufficient to distinguish among six strawberry cultivars (Radmann et al., 2006; Williams et al., 1990).

The phylogenetic relationships of the six strawberry cultivars was based on 228 polymorphic alleles and a dendrogram constructed with unweighted pair group method with arithmetic average (UPGMA) (Fig. 1A). The data matrix showed similarities from 35% (between Praratchatan No.70 and Praratchatan No.72) to 83% (between Praratchatan No.50 and Praratchatan No.70) (Supplementary Table 3). Previous publications of strawberry

cultivars detected similar index values (Morales et al., 2011; Zebrowska and Tyrka, 2003). Based on the similarities, the dendrogram divided the six strawberry cultivars into three groups. Group I was formed by the four cultivars No.329, Praratchatan No.80, Praratchatan No.50, Praratchatan No.70 and group II and III consisted of Akihime and Praratchatan No.72, respectively (Fig. 1A). The low similarity between cultivar No.329 and Praratchatan No.72 could be related to their different origins (Israel and Japan) or the different breeding approaches adapted for these two cultivars (Darrow, 1966). This genetic information can be used to establish family trees and the genetic distance to common ancestors (Morales et al., 2011). For a more detailed study of the genetic diversity with microsatellite-based markers, additional genotyping techniques such as ISSR and AFPL are advisable to provide as much coverage of the genome as possible.

2.2. Metabolite diversity of strawberry leaf

GC-MS analysis of polar and non-polar extracts of strawberry leaf identified 129 metabolites (Supplementary Table 4). The majority of these metabolites (85%) are classified as primary metabolites and comprised sugars, amino acids, intermediates of the TCA cycle and components of the cell membrane/wall. The dendrogram based on this metabolite data showed a very similar grouping compared to the RAPD data (Fig. 1B). Both the clusters for Akihime and Praratchatan No.72 separated the furthest from the Praratchatan No.50 and Praratchatan No.70 clusters, which displayed the least separation. This confirms that the RAPD technique can be applied for the characterisation and certification of strawberry cultivars. Furthermore, the data suggests that the decamer primers bind to parts of the genome responsible for primary metabolism and could be used for genome exploration or marker-assisted breeding (Vallarino et al., 2018).

The principal component analysis (PCA) of the metabolite data showed distinct clusters of biological replicates for each cultivar (Supplementary Fig. 1). Most of the chemical classes contributed equally to the variance of the six cultivars, with the exception of amino acids and the isoprenoids α-tocopherol, phytol and squalene. These metabolites were associated with Praratchatan No.72 and Akihime. Amino acids and isoprenoids with antioxidant properties are important factors for stress response which might give these two cultivars an advantage under unfavourable growth conditions, as well as conferring quality traits (Dixon, 2001; Kliebenstein, 2004; Turhan and Eris, 2009). The phenylpropanoids detected by GC-MS included intermediates of the phenylpropanoid pathway and components of plant cell walls. They were

associated with Praratchatan No.50, Praratchatan No.70 and cultivar No.329. A more detailed metabolomics analysis of the phenylpropanoid superpathway would be necessary to assess the involvement of the phenylpropanoid intermediates in their physiological processes (Deng and Lu, 2017; Kliebenstein, 2004; Winkel-Shirley, 2001). Meanwhile, Praratchatan No.80 has previously demonstrated high resistance to anthracnose, powdery mildew, and two-spotted spider mite (Pipattanawong et al., 2011). The present metabolite data highlighted that this cultivars had higher levels of precursors and components of the cell wall (e.g. myo-inositol, glycerol, glycerol-hexose and fatty acid C26:0) and the lowest levels of catechin and a structural analogue of quercetin. This would indicate that the resistance properties of Praratchatan No.80 are related to the strength/composition of the cell wall (Miedes et al., 2014). Furthermore, Praratchatan No.80 showed higher levels of several mono- and disaccharides including sucrose, glucose, fructose and galactose. In plants sugar signalling plays an important part in regulation of hormone signalling, photosynthesis, source-sink relation and developmental processes, which is evidently different in this cultivar (Akšić et al., 2019; Rolland and Sheen, 2005; Sirijan et al., 2019).

2.3 Metabolite changes throughout strawberry fruit development

The consumer preference and unique leaf chemotypes of Akihime and Praratchatan No.80 suggest a more detailed analysis of the fruit tissue. Samples were collected at eight ripening stages from small green to overripe red fruit (Fig. 2A and B). Fruit samples were subjected to GC-MS and LC-MS analysis and 134 primary and specialised metabolites were identified (Supplementary Table 5 and 6). PCA analysis showed the primary difference between the samples was the fruit ripening as indicated by principle component 1 (x-axis), whereas the second principle component (y-axis) represented the metabolic difference between the two cultivars (Fig. 2C). The score plot highlighted that the variation of the biological replicates was greater in the early ripening stages and that the metabolite composition of the two cultivars was more similar at the red fruit stages.

The change from immature to mature fruit could be observed visually at 16 days after anthesis (Daa) and was reflected in the metabolite data (Fig. 2 and 3). The majority of metabolites (94%) changed during the ripening process and two thirds of these metabolic changes differed between the two strawberry cultivars. A distinct change was detected at 16Daa and affected the polar and non-polar metabolites differently. The PCA of polar extracts showed a grouping of 16Daa with samples at 19-28Daa, whereas an opposite trend was detected for non-polar extracts (Supplementary Fig. 3). This could indicate that at 16Daa, the strawberry

fruit is reprogramming parts of the primary metabolism (e.g. glycolysis, amino acids, TCA cycle) to support the transformation to red ripe fruit, but at the same time maintaining cell membrane/wall processes to support cell enlargement. At 19Daa the strawberry fruit seemed to have reached the genetically defined size and any changes in the texture should be a result of cell wall disorganisation (Schwab and Raab, 2004). This hypothesis is supported by the metabolite data which showed a decrease of fatty acids C16:0, C18:0 and their glycerol esters (Supplementary Fig. 4). This change occurred as a sudden drop at 19Daa in Akihime and as a subtle, steady decrease in Praratchatan No.80. Similar results have been previously reported for the Israeli cultivar Herut as a potential support for oil biosynthesis in the achenes (Fait et al., 2008).

Similar to the fatty acids, amino acids were associated with the immature ripening stages (Supplementary Fig. 3B). The most prominent amino acids were glutamic acid, aspartic acid, asparagine and GABA as previously reported (Burroughs, 1970). These amino acids were decreased throughout ripening, with the exception of glutamic acid which showed no significant change throughout ripening. Previous studies highlighted the importance of amino acids connected to nitrogen assimilation and their involvement in synthesis of other amino acids through the TCA cycle (Galili et al., 2008). All other amino acids followed the same trend of decreased levels from 7Daa to 16Daa followed by an increase until 28Daa. This trend was more pronounced for serine, leucine, alanine and valine in Akihime. Akihime also showed higher levels of amino acids in mature fruits compared to Praratchatan No.80. These changes were expected as the increase of amino acids, such as valine, leucine, isoleucine and alanine, is related to the biosynthesis of volatiles, an important component of strawberry aroma in mature fruit (Perez et al., 1992; Tressl and Drawert, 1973).

A significant, steady increase of mono- and disaccharides was detected throughout ripening in both cultivars (Supplementary Fig. 5). The increase of sugars, especially sucrose, can be expected due to their signalling function for developmental processes (Basson et al., 2010; Hancock, 1999; Jia et al., 2013; Zhang et al., 2011). However, the sucrose accumulation for Praratchatan No.80 commenced at 10Daa, reached the highest levels at 19Daa, followed by a reduction in sucrose levels until the red ripe stage (25Daa) and a slight increase at the overripe stage (28Daa). Contrary to this, Akihime showed a steady accumulation of sucrose levels from 16Daa until the overripe stage. This difference in sucrose accumulation could be related to the significantly higher levels of sucrose in leaf tissue of Praratchatan No.80, leading to an earlier source-sink allocation (Pipattanawong et al., 2011; Sirijan et al., 2019).

For the intermediates of the TCA cycle, no significant change could be detected throughout the ripening process for both cultivars (Supplementary Fig. 5). This result is contrary to previous findings reporting a decrease of organic acids throughout ripening (Fait et al., 2008). However, the present study showed increases and decreases throughout ripening for pathways (glycolysis and synthesis of amino acids and fatty acids) connected through the TCA cycle and demanded regulation of the latter to support these metabolic processes. This particular metabolic phenotype might be a unique feature of Akihime and Praratchatan No.80. The last group of metabolites identified in the current study are polyphenol phytochemicals derived from the phenylpropanoid superpathway and included hydrolysable tannins (ellagitannins), condensed tannins (proanthocyanidins) and flavonoids (Supplementary Fig. 6 and 7). Condensed tannins are mainly produced at the immature stage as they are associated with an astringent flavour and protect the developing fruit from frugivores and other pests. Contrary to this, anthocyanins (e.g. pelargonidin-glucoside) are produced at mature stages to attract frugivors (Almeida et al., 2007; Dixon et al., 2005; Landmann et al., 2007). These general trends could be observed for most of the polyphenol phytochemicals with a few exceptions detected in Praratchatan No.80. This cultivar showed a spike of bis-HHDP-glucosides and proanthocyanidins levels at 13Daa and of kaempferol-glucosides levels at 25Daa. The majority of phenylpropanoid derived compounds refer protection against pathogens and pests and are probably the cause of the superior resistance properties of Praratchatan No.80 (Ahuja et al., 2012; Hébert et al., 2002; Ruuhola et al., 2013).

Polyphenol phytochemicals also contribute to the total antioxidant capacity (TAC), which is an important part of the nutritional quality of strawberries (Giampieri et al., 2013). Previous breeding studies highlighted that TAC is a cultivar specific trait and cultivation conditions have little effect on the nutritional quality of strawberries (Capocasa et al., 2008). The comparison of Praratchatan No.80 to Akihime at the red ripe stage (25Daa) highlighted the 3- to 9-fold higher contents of polyphenols and flavonoids as well as the 2-fold higher contents of pelargonidin-glucoside and salidroside in Praratchatan No.80 (Fig. 4). This suggests that the resistant Praratchatan No.80 also has a higher nutritional quality and therefore provides a good candidate for future breeding programs (Kallscheuer et al., 2019; Pipattanawong et al., 2011; Sirijan et al., 2019).

2.4 Investigation of volatile compounds in the fruit development of two strawberry cultivars.

The aroma of strawberries can be measured using a SPME GC/MS approach and can provide important information on the catabolic processes operating in the fruit (Jetti et al., 2007; Pérez et al., 2002; Yamashita et al., 1977). Akihime and Praratchatan No.80 were analysed at four ripening stages (green, white, red, over-ripening) with a SPME protocol adapted from de Boishebert et al. (2006) for freeze-dried tissue. A total of 74 volatile compounds were identified and showed a clear separation of ripening stages and cultivars (Fig. 5A, Supplementary Fig. 8, and Supplementary Table 7). The least separation was observed between 16 and 22Daa for both ripening stage and cultivar types. The loading plot highlighted that alcohols, aldehydes and terpenes were associated with the immature green stage and esters with the mature red stage (Fig. 5B). The same compositional differences have been previously reported for strawberry and have been associated with a reduced activity of ester-forming enzymes at the immature stage (Forney et al., 2000; Jetti et al., 2007; Perez et al., 1992; Yamashita et al., 1977). This can be confirmed by the present data, as mainly methyl esters were detected in the immature stage. Akihime showed significantly higher levels (3- to >40-fold) of saturated fatty acid esters at both the immature and mature stage (7 and 28Daa). Praratchatan No.80 had higher levels of unsaturated fatty acid esters at 28Daa. Furthermore, at 7Daa alcohols were higher in Akihime and aldehydes and GPP derived volatiles in Praratchatan No.80. These volatile profiles suggest very different metabolic regulation in the two cultivars and correlates with the fatty acids levels detected. At 28Daa, Akihime and Praratchatan No. 80 showed lower levels of the saturated and unsaturated fatty acids, the respective precursors to the volatiles detected (Sanz et al., 1997; Schwab and Schreier, 2002). As mentioned earlier, Akihime showed a sudden decrease of C16:0 and C18:0 at 16 to 19Daa, which might be the result of an induced oxidation of fatty acids. The products of this reaction can be used as precursors for biosynthesis of volatile esters, which were higher in Akihime and suggest genetic differences in aroma formation between the two cultivars (Osorio et al., 2010).

3. Conclusions

The results obtained in the present study illustrated that RAPD markers can easily distinguish between strawberry cultivars with different degrees of genetic relationship. Furthermore, the primers used for RAPD generated datasets which displayed a very close reflection of the metabolic data. The combination of these two techniques provide strong certification and characterisation of Thai strawberries and can be applied to assess the strawberry germplasm collection for future breeding efforts and true-to-type confirmation for

producers. The metabolomics approach which focuses on the end products of cellular regulation could overcome the practical difficulties associated with working with octoploid crops such as strawberry. The analysis of the ripening stages provided insight into the different metabolic regulations of Akihime and Praratchatan No.80. In combination with genetic markers, this information will facilitate breeding approaches for specific traits in new strawberry cultivars in Thailand.

4. Experimental

4.1 Plant and growth conditions

Six strawberry cultivars, *Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier (family *Rosaceae*) Praratchatan No.50, Praratchatan No.70, Praratchatan No.72, Praratchatan No.80, No.329 and Akihime, were cultivated at The Royal Project Foundation, Chiangmai, northern Thailand (latitude: 18.812369, longitude: 98.884381). Six biological replicates of each cultivar were maintained in the greenhouse under approximately 17.5°C and 75% relative humidity with 16h light and 8h darkness. Young leaf samples were collected in triplicate ~1 month after planting. Fruit samples for Praratchatan No.80 and Akihime were collected at eight different development stages: small green fruit stage 7 day after anthesis (Daa), 10 Daa (large green fruit), 13 Daa (green-white fruit), 16 Daa (white fruit), 19 Daa (turning fruit), 22 Daa (red fruit), 25 Daa (red-ripening fruit) and 28 Daa (over-ripening fruit) (Fig. 2). After harvest, three fruits of each biological replicate were washed in water, cut into cubes, pooled and immediately frozen in liquid nitrogen. All samples were kept at -80°C until analysis.

4.2 DNA extraction and RAPD analysis

Genomic DNA was isolated from young leaf samples using Qiagen DNeasy plant mini kit (Qiagen Ltd., Crawley, UK). After confirmation of the DNA quality and quantity, all samples were diluted to ~50ng/µl and stored at -20°C. RAPD with Illustra puReTaq Ready-to-Go PCR beads (GE Healthcare) was performed with 25 different primers (10µM, Operon Technologies, USA), ten nucleotides in length (Supplementary Table 1). The PCR reaction was carried out with an initial denaturation step at 94°C for 4 min, followed by 35 cycles at 94°C for 30 sec, 35°C for 30 sec, 72°C for 2 min and 72°C for 5 min using a Thermal cycler (Bio-Rad T100^{TM}, USA). All the reactions were repeated at least twice to check the reproducibility of banding patterns. Non-reproducible bands were excluded.

4.3 Extraction of metabolites form leaf and fruit tissue

Freeze-dried samples from leaf (~0.5 g) and fruit (~10 g) were ground into a fine powder. Samples were weighed (10 ± 0.5mg) and extracted with a methanol–chloroform protocol as previously described (Nogueira et al., 2013). Aliquots of the polar and non-polar phase were analysed separately.

4.4 GC-MS analysis

The aliquots of the polar phase of leaf (150µl) and fruit (20µl) were dried down with the internal standard $_{d4}$-succinic acid (5µg). Aliquots of the non-polar phase (700µl) were dry down with $_{d27}$-myristic acid (10µg). All samples were derivatised immediately before analysis in splitless mode with an 7890A gas chromatography (GC) system coupled with a mass spectrometer (MS) 5795C MSD (Agilent Technologies, Inc.) as previously described (Enfissi et al., 2010). Identification of metabolites was performed with AMDIS (V2.71) based on a retention time, retention index and mass spectrum comparison to an in-house library (Supplementary Table 5).

4.5 LC-MS analysis

An aliquot of the polar fruit extract (200µl) was filtered using a syringe filter (nylon, 0.45 µm). Internal standard was added to an aliquot (100µl) of the filtrate and subjected to analysis with an Agilent 6560 Ion Mobility Q-TOF coupled to an Agilent 1290 Infinity II (Agilent Technologies, Inc.) in positive and negative electrospray ionisation mode (100-1700m/z, 0.9spectra/second). Samples were separated with a Zorbax column (Agilent Technologies, Inc.) and gradient of solvent A (water and 0.1% formic acid) and solvent B (2.5% water in acetonitrile and 0.1% formic acid) at a flow rate of 0.3ml/min. The gradient started with 95% A for 1min, followed by a linear gradient to 70% A at 6min and 2% A at 7.5min, which was held for 1.5min. The gradient was then returned to the start conditions 95% A at 10.5min and the column was re-equilibrated for 1.5min. The source settings included nozzle and capillary voltages at -500V/500V and 4000V, nebuliser gas (nitrogen) at 35psi, dry gas at 5l/min and 325°C and sheath gas at 12l/min and 275°C. Calibration was performed during each run to a reference solution. For identification of compounds with a UV/VIS spectrum, representative samples were separated under the same chromatographic conditions as described above and the eluent analysed with a DAD module (scan mode 200-600nm) before MS analysis. Data analysis was performed with Agilent Profinder (V10.0 SP1, Agilent Technologies, Inc.) with a retention time tolerance 0.2min and mass tolerance 5ppm for peaks

>1000 counts. Metabolites were identified through authentic standards and comparison of MS/MS spectra to previous strawberry publications (Aaby et al., 2007; Guo et al., 2012; Lopes-da-Silva et al., 2002).

4.6 SPME analysis

Freeze-dried strawberry powder (100mg) of 7, 16, 22 and 28 Daa was suspended in 10% (w/v) sodium chloride (2ml) in a screw cap head space vial (5ml) and $_{d3}$- β, β, β-acetophenone (50µl of a 1ppm stock solution) added to each aliquot. Sample were sonicated for 1min before a modified version of the previously published analysis by SPME-GC-EI-MS (de Boishebert et al., 2006).

Volatiles were identified through authentic standards, NIST11 database (2011) and comparison of retention index and mass spectrum of previous publications (see Supplementary Table 4).

4.7 Data Processing and Statistical Analysis.

For RAPD markers analysis, the presence or absence of each PCR product for each marker was scored as "1" or "0", respectively. A pairwise similarity matrix was generated using the Nei-Li similarity index (Nei and Li, 1979). The phylogenetic tree was dependent on bootstrap analysis with 5000 replicates. A dendrogram was constructed based on the similarity matrix data by applying the unweight pair-group method with arithmetic average (UPGMA) cluster analysis using Free tree and Tree view X 1.0 software (Pavlícek et al., 1999)

For metabolite analysis, all metabolites were quantified relative to the respective internal standard. Data was processed with SimcaP (13.0.3.0, Umetrics) for PCA and hierarchical analysis and Metaboanalyst (Xia and Wishart, 2016) for heatmaps, time-series analysis and ANOVA. The metabolite differences at 25Daa were calculated with Student *t*-test ($P<0.05$) and presented as a pathway display created with in-house software.

Declaration of interest

The authors declare no conflict of interest.

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Availability of data and materials

Processed data is available in the manuscript and appendices. Unprocessed data can be accessed at doi:10.17632/df9rwnnnzd.2.

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Figures

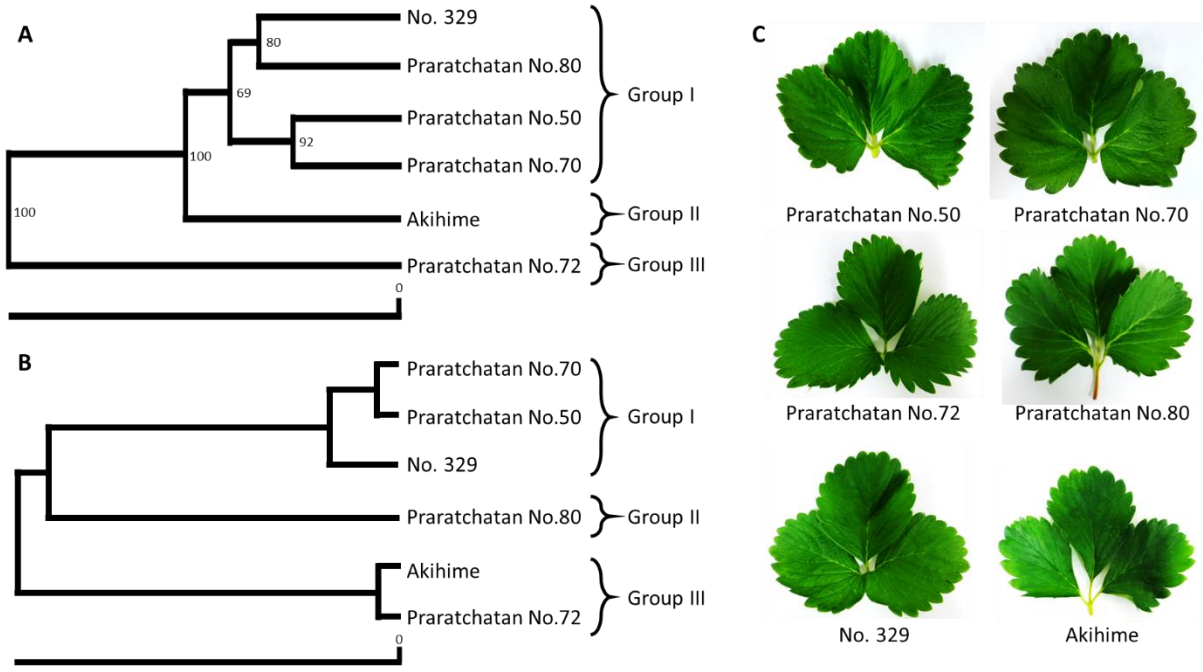

Fig. 1. Dendrograms of six strawberry varieties based on their genetic (A) and metabolic (B) similarities. The genetic information is based on the degree of band sharing of 228 polymorphic alleles. The metabolic data comprises 129 metabolites identified in polar and non-polar leaf extracts by GC-MS. (C) Representative leaf of six strawberry cultivars.

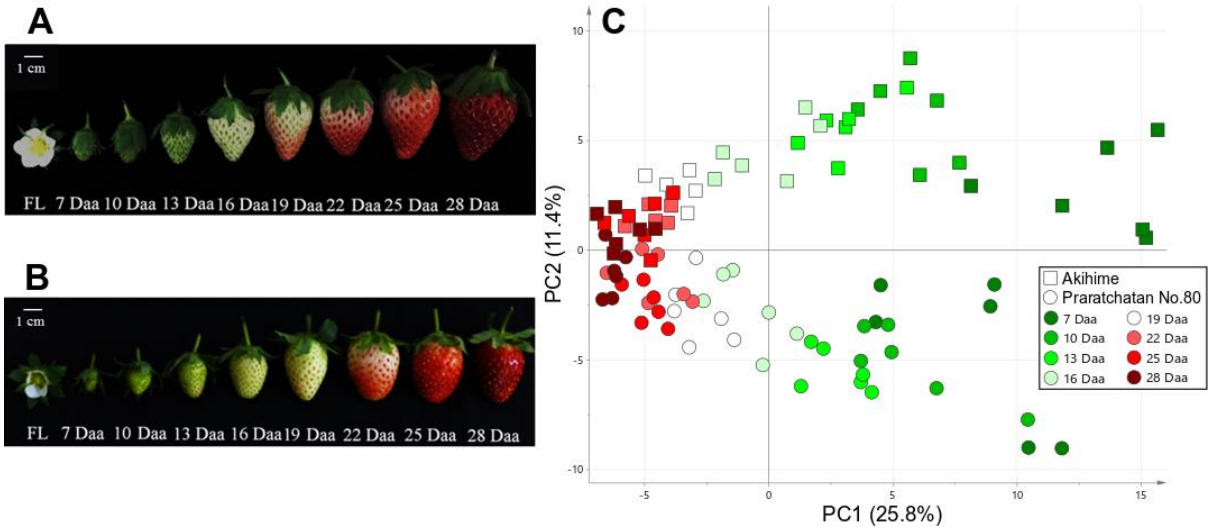

Fig. 2. Stages of fruit development from flower (FL) to overripe fruit of strawberry cultivar Akihime (A) and Praratchatan No.80 (B). Stages are labelled as days after anthesis (Daa).

PCA analysis of metabolite composition of Praratchatan No.80 (circles) and Akihime (squares) at eight different fruit ripening stages (C). Metabolites of polar and non-polar extracts were analysed by GC-MS and LC-MS. Data includes 134 identified metabolites including primary and specialised metabolism. Analysis comprised six biological replicates, which are displayed individually.

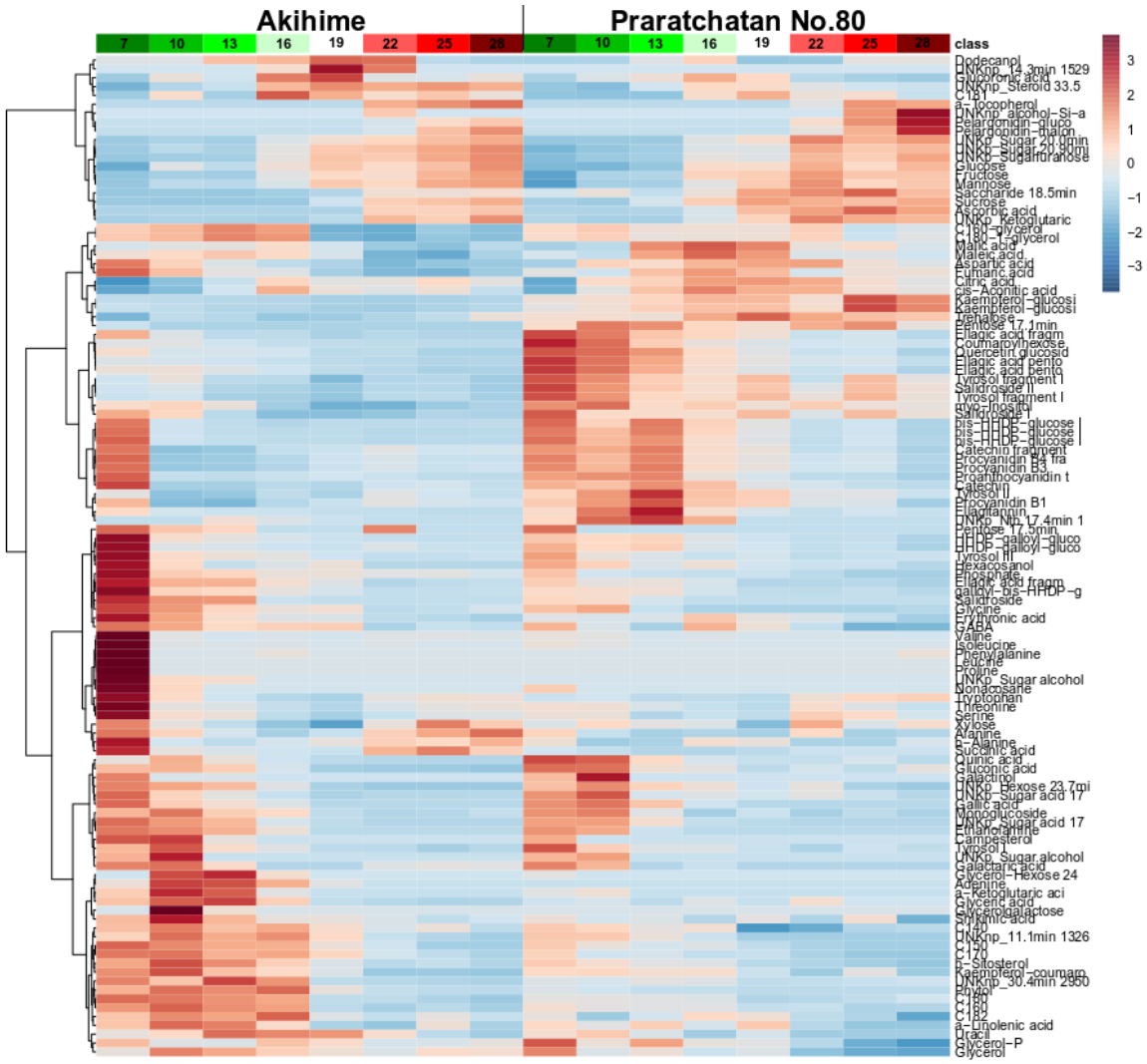

Fig. 3. Heatmap of metabolites detected in strawberry fruits of Praratchatan No.80 and Akihime. Metabolites were analysed by GC-MS and LC-MS from polar extracts and by GC-MS from non-polar extracts. Ripening stages are displayed as 7, 10, 13, 16, 19, 22, 25, 28Daa. Only significant compounds, as determined by two-way ANOVA analysis, are displayed. Biological replicates are displayed individually. A more detailed version of this figure is available as Supplementary Fig. 3.

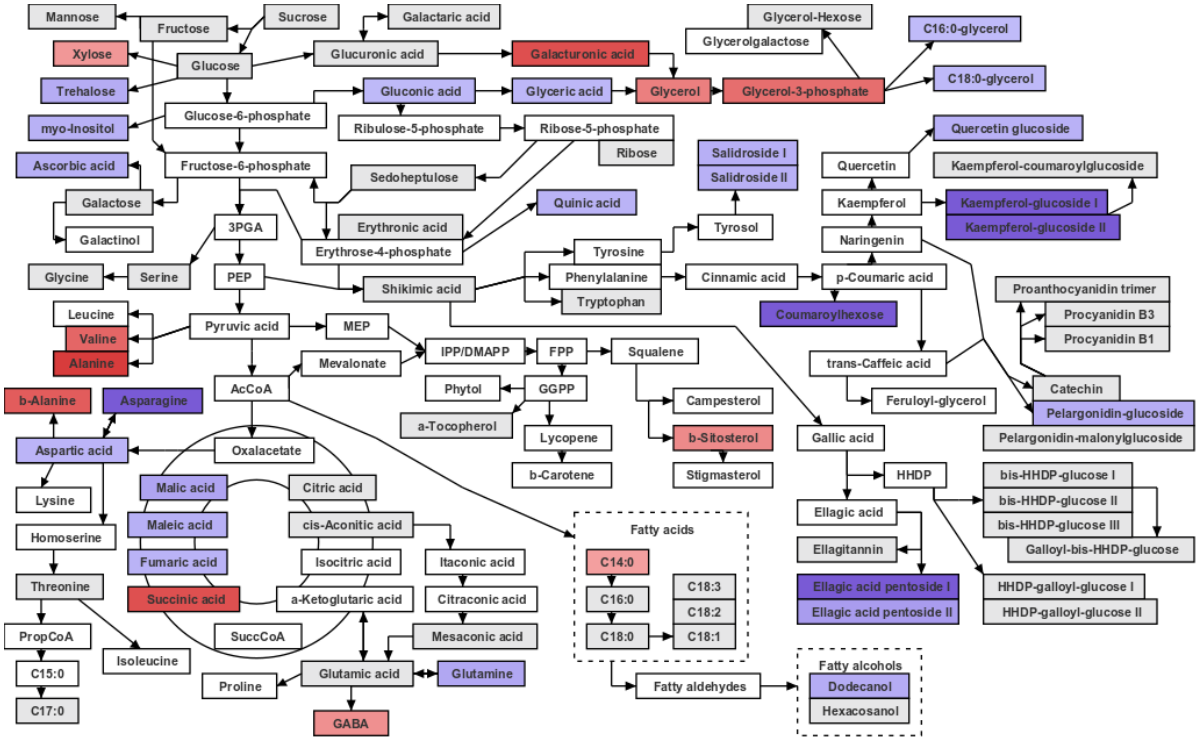

Fig. 4. Metabolite pathway display shows significant difference between Praratchatan No.80 and Akihime at 25Daa. Changes of metabolites are displayed as ratio >1 (blue), <1 (red), no change (grey) and not detected (white). Analysis comprised six biological replicates per cultivar.

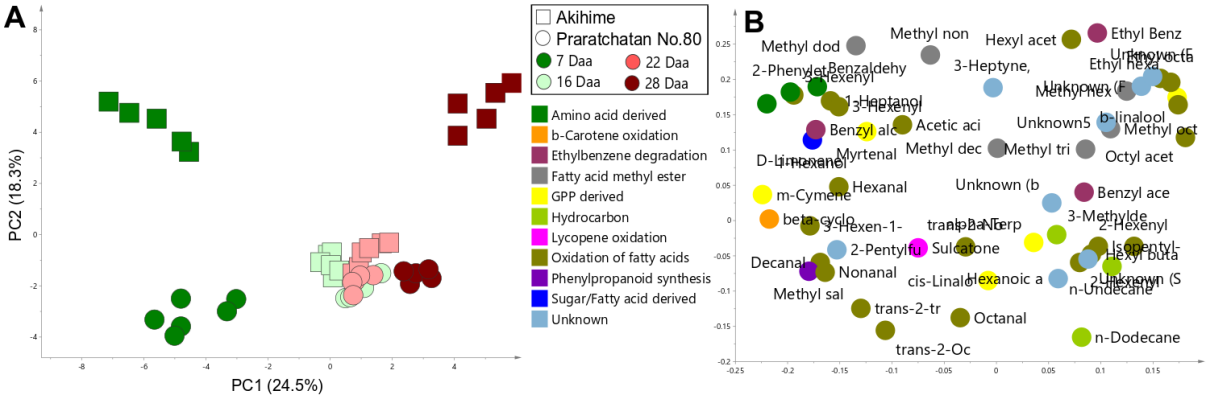

Fig. 5. PCA analysis of volatile analysis of Akihime and Praratchatan No.80. Freeze-dried tissue was analysed by SPME-GC-MS. Samples and metabolites are displayed as score plot (A) and loading plot (B). Six biological replicates are displayed individually.