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Targeted quantitative proteomic investigation employing multiple reaction monitoring on quantitative changes in proteins that regulate volatile biosynthesis of strawberry fruit at different ripening stages

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ABSTRACT

A targeted quantitative proteomic investigation employing the multiple reaction monitoring (MRM, SRM) technique was conducted on strawberry fruit at different development stages. We investigated 22 proteins and isoforms from 32 peptides with 111 peptide transitions, which may be involved in the volatile aroma biosynthesis pathway. The normalized protein abundance was significantly changed in coincidence with increased volatile production and advanced fruit maturities. Among them, alcohol acyltransferase (AAT), quinone oxidoreductase (QR), malonyl Co-A decarboxylase, (MLYCD), pyruvate decarboxylase (PDC), acetyl Co-A carboxylase (ACCase), and acyl Co-A synthetase (ACAs) were increased significantly. Several alcohol dehydrogenases (ADHs), and 3-oxoacyl-ACP synthase were significantly decreased. Furthermore, the expression of seven genes related to strawberry volatile production was also investigated using real-time qPCR. Among the tested genes, *QR*, *AAT*, *ACCase*, *OMT*, *PDC* and *ADH* showed increased up-regulation during fruit ripening, while 3-isopropylmalate dehydrogenase (*IMD*) decreased. Strong correlation between quantitative proteomic data and gene expression suggested that AAT, QR, ACCase, and PDC played critical roles in volatile biosynthesis of strawberry during fruit ripening. Poor correlation between protein abundance and gene expression of ADH was found.

1. Introduction

Strawberry (*Fragaria* × *ananassa*) fruit is the most consumed berry fruit crops worldwide and is valued for its nutritional and flavor quality. The total worldwide production is estimated at 4.59 million tonnes in 2011 [1]. The eating quality of fresh strawberry fruit, which includes appearance, color, texture, flavor (taste and aroma) and nutritional value, is influenced by both genetic and environmental factors. Strawberries are generally recognized to be a very perishable fruit and have a short shelf life [2]. During ripening, strawberry fruit shows rapid changes in color, formation of volatile compounds, increase of anthocyanin pigments and decrease of firmness and acidity that determine postharvest quality. These dynamic changes influence the consumer's perception of fresh strawberry fruit quality. Researches on strawberry fruit flavor have identified more than 300 volatile aroma compounds during ripening; the majority are esters, alcohols, aldehydes, terpenes and acids [3]. Harvest maturity plays a pivotal role in volatile development of strawberry fruit and there is a rapid transition between unripe and ripe fruit that occurs within 1 or 2 days in the field [4]. C_6 aldehydes were identified as the major compounds in immature white fruit, while esters and furanone are present in three quarter red or fully red fruit [5]. These flavor volatiles increase many folds as fruit ripens on the plant [4]. After harvest, flavor volatiles in strawberry fruit continue to increase, but this increase is greatly diminished if fruit is harvested prior to fully red-ripe [4]. As strawberry is a non-climacteric fruit, postharvest ripening and flavor development do not respond to treatment with ethylene [2].

Both branched chain amino acid and straight chain fatty acid metabolism have been proposed to be involved in volatile aroma production in fruit, however, the control mechanisms and regulatory steps are not fully understood. The involvement of alcohol acyltransferase (AAT) in ester formation of many fruit has been reported [6]. AAT plays a critical role in production of esters in strawberry fruit during ripening. It increases at the transcript level during fruit ripening [7]. However, the origin of precursors leading to ester formation has not been determined. The furanones, furaneol (4-hydroxy-2,5-dimethyl-3-furanone) and mesifurane (4-methoxy-2,5-dimethylfuran-3-one) are also key volatile compounds in strawberry and 3(*2H*)-furanone compounds have been reported in strawberry fruit [8,9]. Furaneol has been described as having a "sweet" and "burnt sugar" aroma and its methylation to mesifurane is

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mediated by an O-methyltransferase (*FaOMT*), that increases in activity during fruit ripening [10]. The biosynthesis of furanones is controlled by FaOMT, a gene that determines the natural mesifurane content in strawberries [11,12]. A study of quantitative trait loci (QTL) detected seventy QTLs covering 48 different volatiles, with several of them being stable over time and mapped as major QTLs [11]. Loci controlling γ-decalactone and mesifurane synthesis were mapped as qualitative traits. It demonstrated that one homolog of the O-methyltransferase gene (FaOMT) is the locus responsible for mesifurane content [11].

Using advanced molecular biological technologies, strawberry genes that were differentially expressed during fruit ripening were identified [13–15]. Alcohol acyl transferase (AAT) and AAT2 genes were shown to play a crucial role in strawberry fruit volatile production [7,16]. Gene expression related to ethylene reception [17], firmness [18], allergens [19], formation of γ-decalactone [20] and anthocyanin biosynthesis [21–23] during strawberry fruit ripening and senescence have also been investigated. Strawberry flavor including sweetness and consumer liking is influenced by genetic and environmental factors [24].

Metabolomic analysis of strawberry receptacle and achene tissues during fruit development and ripening revealed that both metabolic synchrony and speciation exist in strawberry fruit [25,26]. In comparison with biochemical and physiological studies using genomic and molecular approaches, very few data are available at the proteomic level to provide insight into volatile biosynthesis and regulation during strawberry fruit ripening.

Proteomics is an important molecular approach in fruit research to reveal biomarkers for breeding and key regulatory mechanisms in relation to fruit ripening, storage shelf-life, nutrition and flavor quality [27,28]. A quantitative proteomic research using a dimethylation of peptides labeling technique combined with off-line OFFGEL gel-electrophoresis (OGE) was applied to determine quantitative changes in proteins related to strawberry fruit development and ripening [29]. Proteins linked to metabolic processes such as flavonoid biosynthesis, volatile production, antioxidants and redox enzymes, stress and ethylene responses were identified and quantified. Two alcohol acyltransferases (AATs), two quinone oxidoreductases and an O-methyltransferase were related to volatile biosynthesis in both 'Honeoye' and 'Mira' strawberries. In addition, two pyruvate decarboxylases (PDC) in 'Honeoye' and 'Mira' and acyl-CoA synthetase in 'Mira' increased [29]. These significant increases of proteins can be explained by the high demand for acyl-CoA for ester and fatty acid biosynthesis during strawberry fruit ripening. This work provides initial proteomic evidence that QR, AAT and PDC become more abundant concurrent with rapid volatile production in ripening strawberry fruit.

In order to reveal the regulating factors responsible for fruit volatile bio-synthesis and fruit ripening, new technologies are required. Among the new proteomic techniques, selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) using a triple quadrupole MS or a quadrupole ion trap instrument, has been developed and emerged as a targeted quantitative proteomic technology for detection and accurate quantification of specific, predetermined sets of proteins in a complex biological sample [30–32]. MRM transitions which are the sets of precursor and fragment ion masses of a given peptide, can be established and determined. With the success of the establishment of transitions, MRM study can be used to investigate the involvement of proteins in metabolic pathways [32]. Over the conventional LC/MS analysis, MRM has higher sensitivity and broader dynamic range (up to five orders of magnitude). There have only been a few reports available on application of the MRM technique to quantitatively investigate fruit proteins in metabolomic pathways. We conducted a MRM study to investigate the proteins involved in flavonoids biosynthesis pathway of strawberry fruit at different ripening stages [33]. In addition, polyphenol oxidases in loquat fruit [34] and monodehydro-ascorbate reductase in apple fruit [35] were also reported.

The goal of this study is to combine OGE isoelectric focussing fractionation with LC–MS/MS analysis to further identify complex strawberry fruit peptides. We then designed, evaluated and established transitions for MRM studies for 22 protein targets proposed by previous studies to be involved in strawberry volatile production in order to investigate their role in volatile regulation. Further, we combined volatile characterization with gene transcript abundance analysis and quantified protein abundance to provide new insights into regulation of volatile production in strawberry fruit during ripening.

2. Experimental procedures

2.1. Plant materials

The harvesting and handling of strawberries (*Fragaria* × *ananassa*, Cv. 'Mira' and 'Honeoye') fruit was published in a previous work. From each harvest, at least 300 g of fruit was harvested for each sample [29].

2.2. Protein extraction and quantification

Protein was extracted and purified from ground fruit samples (1.25 g) using a modified phenol extraction followed by ammonium acetate-methanol precipitation [35] and [36]. Briefly, the sample was homogenized in 5 mL of ice-cold extraction buffer containing 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl, pH 8.5, 50 mM EDTA, pH 8.5, 1% (m/v) insoluble polyvinylpolypyrrolidone (PVPP), 40 mM dithiothreitol (DTT), and an equal volume of ice-cold Tris-buffered phenol (pH 8.0) at 6000 rpm for 60 s (Sentry™ Microprocessor with Cyclone I.Q.^{2}, Virtis, SP Industries, Gardiner, NY, USA). After centrifugation at 10,000 ×*g* for 15 min at 4 °C to obtain a clarified supernatant, the phenol phase was re-extracted and combined. The pooled phenol phase was precipitated with five volumes of ice-cold solution (0.1 M ammonium acetate in 100% methanol) and incubated overnight at −20 °C. The resultant pellet was obtained after a centrifugation at 14,000 × *g* for 15 min at 4 °C, and then was washed twice with ice-cold methanol, then twice with ice-cold acetone containing 20 mM DTT. The final protein extract was dissolved in 500 μL of IEF buffer [7 M urea, 2 M thiourea, 40 mM DTT and 1% (v/v) IPG buffer (pH 3–10)] and centrifuged at 12,000 × *g* for 5 min at room temperature.

2.3. Tryptic in-solution digestion and desalting

Digestion and desalting procedures were carried out as previously described [35]. Briefly, protein samples were diluted with triethylammonium bicarbonate (TEAB) to a final concentration with 1 M urea or less, then reduced with DTT for 30 min at 30 °C and alkylated with iodoacetamide for 60 min at room temperature in the dark. Proteins were digested with sequencing-grade modified trypsin (Promega, Madison, WI) overnight at 37 °C with a final trypsin to substrate ratio of 1:50 (w/w). The peptides were desalted using Oasis HLB cartridges (Waters, Milford, MA).

2.4. OFFGEL fractionation

The p*I*-based separation of total strawberry total protein digest (peptides) was conducted on a 3100 Off-gel Fractionator (Agilent Technologies, Palo Alto, CA, USA) with 24-wells according to the manufacturer's protocol with modifications [35]. After the peptide OGE, the peptides (volume between 50 and 200 μL) collected from each well were desalted as described previously, dried and re-dissolved in 2% (v/v) ACN and 1% (v/v) formic acid just prior to LC/MS analysis.

2.5. LC/MS/MS and data analysis

The detailed setting for LC/MS was described in previous study [35]. Nano-LC/MS was performed on an Ultimate nano-flow system (Dionex, Sunnyvale, CA, USA) and a hybrid triple quadrupole linear ion trap