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Quantitative changes in proteins responsible for flavonoid and anthocyanin biosynthesis in strawberry fruit at different ripening stages: A targeted quantitative proteomic investigation employing multiple reaction monitoring

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ABSTRACT

To better understand the regulation of flavonoid and anthocyanin biosynthesis, a targeted quantitative proteomic investigation employing LC-MS with multiple reaction monitoring was conducted on two strawberry cultivars at three ripening stages. This quantitative proteomic workflow was improved through an OFFGEL electrophoresis to fractionate peptides from total protein digests. A total of 154 peptide transitions from 47 peptides covering 21 proteins and isoforms related to anthocyanin biosynthesis were investigated. The normalized protein abundance, which was measured using isotopically-labeled standards, was significantly changed concurrently with increased anthocyanin content and advanced fruit maturity. The protein abundance of phenylalanine ammonia-lyase; anthocyanidin synthase, chalcone isomerase; flavanone 3-hydroxylase; dihydroflavonol 4-reductase, UDP-glucose:flavonoid-3-O-glucosyltransferase, cytochrome c and cytochrome C oxidase subunit 2, was all significantly increased in fruit of more advanced ripeness. An interaction between cultivar and maturity was also shown with respect to chalcone isomerase. The good correlation between protein abundance and anthocyanin content suggested that a metabolic control point may exist for anthocyanin biosynthesis. This research provides insights into the process of anthocyanin formation in strawberry fruit at the level of protein concentration and reveals possible candidates in the regulation of anthocyanin formation during fruit ripening.

Biological significance
To gain insight into the molecular mechanisms contributing to flavonoids and anthocyanin biosynthesis and regulation of strawberry fruit during ripening is challenging due to limited molecular biology tools and established hypothesis. Our targeted proteomic approach

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employing LC-MS/MS analysis and MRM technique to quantify proteins in relation to flavonoids and anthocyanin biosynthesis and regulation in strawberry fruit during fruit ripening is novel. The identification of peptides and proteins provided reliable design and validation of quantitative approaches using SRM on targeted proteins proposed involved in strawberry fruit. Our data revealed the identifying candidate proteins and their quantitative changes in relation to fruit ripening and flavonoids and anthocyanin biosynthesis and regulation. More importantly, this quantitative proteomic data is also compared with chemical analysis to reveal possible control levels of this important quality trait.

Although, MRM approach is not new in plant biology research, the application has been very rare. This is the first systematic multi-targeted interrogation of the possible regulation of entire pathway of flavonoids and anthocyanin biosynthesis in strawberry fruit at different ripening stages using quantitative MRM technique on mass spectrometry. Our results demonstrate the power of targeted quantitative mass spectrometry data for analysis of proteins in biological regulation. These results indicate that distinct and diverse control of flavonoids and anthocyanin biosynthesis mechanisms at metabolism and proteins levels. This important and complementary knowledge will be useful for systematically characterizing the flavonoids and anthocyanin biosynthesis pathway of any fruit/plant species.

1. Introduction

Strawberry (*Fragaria* × *ananassa*) is an important fruit crop worldwide and popular to the consumers due to its nutritional and flavor quality. During fruit ripening, strawberry fruit produces only a minimal amount of ethylene and without either a significant burst in respiration or ethylene production. However, it undergoes rapid changes in color from white to red, and an increase in the formation of flavor volatiles and a decrease of fruit firmness and acidity [1]. Together these changes in fruit composition strongly influence postharvest quality and influence the consumer's perception of the quality of fresh strawberry fruit.

Significant efforts using molecular biological tools to investigate strawberry fruit ripening and senescence have been reported. Several studies identified differentially expressed genes in strawberry fruit during ripening [2–4]. Applying DNA micro array technology, an alcohol acyl transferase (SAAT) gene was identified and found to be closely related to fruit volatile production [5]. Other studies conducted on strawberry fruit during ripening identified genes related to ethylene reception [6], fruit firmness [7], allergens [8] and anthocyanin biosynthesis [9–11]. At the metabolomic level, a metabolic synchrony and specialization in both the achenes and receptacles of strawberry fruit during development and ripening were reported [12].

During fruit development and ripening, strawberry fruit showed two phases of increased flavonoid biosynthesis with major flavonoid compounds decreasing in red ripe fruit as the content of flavonoids, except for flavonols [13]. In most strawberry cultivars, the increase of pigmentation of the receptacle tissue is mainly due to the accumulation of mono-glycosylated pelargonidin and a small amount of glycosylated cyanidin [12,14]. New approaches in metabolomics confirmed that the major pigment change in strawberry was the significant increase in pelargonidin-3-*O*-glucoside (Pg3 glc) and cyanidin-3-glucoside (Cy3 glc) [15].

Investigations at the transcript level reveled a significant increase in phenylalanine ammonia-lyase (PAL), anthocyanidin synthase (ANS), chalcone isomerase (CHI); chalcone synthase (CHS), and flavanone 3-hydroxylase (FNS) in the flavonoid biosynthetic pathway, while a down and up expression pattern for the phenylpropanoid genes, FaPAL, cinnamic acid 4-hydrolyase (FaC4H) and *p*-coumarate CoA ligase (Fa4CL) which showed a peak level of transcripts at the color transition stage but a lower level in white fruit [16]. Gene expression of the anthocyanidin glucosyltransferase (*FaGT1*) increased significantly during fruit development from white to pink and red fruit, which correlated to fruit ripening [11]. But a negative regulation of auxin on gene expression of the *FaGT1* led to a significant decrease of pelargonidin-3-*O*-glucose malonyl and pelargonidin *O*-glucoside. These results demonstrated that anthocyanidin glucosyltransferase (FaGT1) may play a crucial role at the branch point in the pathway to influencing metabolite flow into the biosynthesis of anthocyanins versus other flavonoids in strawberry fruit [11].

Although significant efforts have been made at the both biochemical and physiological level using genomic [11] and molecular [17,18] tools, studies at the proteomic level to address flavonoid biosynthesis and its regulation during strawberry fruit ripening have been rare.

Proteomics has become one of the most important molecular tools in fruit research to understand physiological regulatory components and to identify biomarkers for breeding designed to improve shelf-life, nutrition and flavor quality [19,20]. A previous proteomic study on strawberry fruit employed 2-D gel based technologies to evaluate hundreds of proteins during fruit ripening [21]. Although two-dimensional electrophoresis (2-DE) coupled with mass spectrometry is still a powerful approach for global protein identification and quantification [22,23], it suffers from low throughput and is limited in its ability to detect of proteins with extreme *pI* and molecular weight. Therefore only a limited numbers of proteins have been identified. Recently we applied a quantitative proteomic technique involving dimethylation labeling of peptides combined with off-line OGE (OFFGEL gel-electrophoresis) to reveal the quantitative changes in the strawberry proteome during fruit ripening. Using this approach, significantly changed proteins associated with flavonoid biosynthesis, volatile production, antioxidant and redox enzymes, response to stress and ethylene biosynthesis were identified [24]. In relation to the flavonoid biosynthesis