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Quantitative proteomic changes in development of superficial scald disorder and its response to diphenylamine and 1-MCP treatments in apple fruit☆

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ABSTRACT

Superficial scald is a significant physiological disorder causing economic loss to the apple and pear fruit industry. Despite treatments to prevent the scald, fundamental biochemical knowledge about the injury is not completely known. To investigate the protein changes in association with scald development during storage and in response to treatments of diphenylamine and 1-MCP, two quantitative proteomic experiments employing stable isotope labeling by peptide dimethylation were conducted on two scald susceptible cultivars 'Cortland' and 'Red Delicious'. Apples were untreated or treated with 2 g L^{-1} DPA or with/without 1 μL L^{-1} 1-MCP, then stored at CA (3.0 kPa O_2 + 1.0 kPa CO_2) at 0–1 °C for up to 7 months. Among the quantified proteins, 428 proteins were common to both cultivars and 110 increased in abundance and 48 decreased during the onset of scald development. Fifty seven and 67 proteins changed significantly in abundance in response to DPA and 1-MCP treatments after 4 and 7 months storage, respectively. When combining the results from both cultivars, 14 proteins increased in abundances and 4 proteins decreased in abundance with scald development but inhibited and enhanced by both DPA and 1-MCP treatments, respectively, which were proposed to be in association with scald development of apples. Our results reveal and confirm that antioxidant and redox system, phenylpropanoid metabolism, ethylene biosynthesis, allergens, sulfur amino acids containing proteins and program cell death have direct link to the scald development.

1. Introduction

Superficial scald is a physiological disorder causing brown or black patches on fruit skin that appears during or after storage on certain cultivars of apples and pears. Scald reduces significantly the market quality of fruit and causes economic losses for the tree fruit industry. Over the years, several hypotheses have been established to explain the development of superficial scald (Lurie and Watkins, 2012). The hypothesis that α-farnesene oxidation products cause cell damage resulting in skin browning has been proposed and researched (Rowan et al., 1995; Whitaker, 2004; Whitaker et al., 1997). A dramatic rise in α-farnesene synthesis occurs shortly after apples were placed in storage, and oxidation of the accumulated α-farnesene proceeds rapidly after approximately 6 to 8 weeks, particularly in air-stored fruit (Rowan et al., 1995). Investigations of metabolic events in apple and pear fruit revealed that the dynamic changes in α-farnesene and its oxidation products, conjugated trienols (CTs) in apple fruit during storage (Whitaker et al., 1997). It was suggested that scald may result from more general oxidative stress, perhaps triggered by disruption of mitochondrial electron transport at low temperature and the consequent production of superoxide (Whitaker, 2004). A novel and accurate model based on CTols accumulation dynamics (dCTols/dt) during early stages of storage (<50 days) was proposed to be used to predict scald development in 'Granny Smith' apples (Giné Bordonaba et al., 2013).

Over more than 50 years, a commercial prevention has been to apply DPA (diphenylamine) or ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline), antioxidants to prevent this disorder (Huelin, 1968; Smock, 1957, 1961). However, DPA application is not permitted in several European countries and fruit treated with the antioxidant are not permitted to be imported. Registration of DPA may be phased out in countries outside of Europe as well

☆ Use of trade names does not imply endorsement of the products named or criticism of similar ones not named.

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(Calvo and Kupferman, 2012). The European Union withdrew authorization for plant protection products containing ethoxyquin in 2009. A continuing registration of ethoxyquin in the United States is not assured since European pear is the only horticultural crop registered for postharvest use (Wang, 2016). Other storage procedures and treatments to minimize this disorder have also applied, for example, low oxygen storage and application of 1-methylcyclopropene (1-MCP), a widely registered inhibitor of ethylene perception (Lurie and Watkins, 2012). Research has shown that while these treatments have reduced the severity, they have not been as effective as DPA. Both application of DPA and low oxygen storage suggest that scald development is not only an oxidative process related disorder, but also an ethylene-dependent process. Application of DPA inhibits CTs and 6-methyl-5-heptene-2-one (MHO) formation in apples (Zupan et al., 2013). Application of 1-MCP is an alternative to current chemicals used to control storage scald in apples (Fan et al., 1999).

Untargeted metabolic profiling in apple fruit peel tissue revealed a large group of triterpenoids similar to ursolic acid and ß-sitosterol were associated with scald (Rudell et al., 2009). Another metabolomic approach identified a divergence of group metabolites after 30 days of cold storage in apple peels which showed different levels of α-farnesene oxidation products, methyl esters, phytosterols, and other compounds in association with chloroplast integrity and oxidative stress response (Leisso et al., 2013). While, a targeted metabolomic study indicated that α-farnesene and its oxidized products may act as a signal system and polyphenol compounds and programmed cell death may be the basis for the disorder development (Busatto et al., 2014). At the genomic level, an (E, E)-α-farnesene synthase gene (AFS1; GenBank accession number AY182241) from "LawRome" apple peel tissue was cloned (Pechous and Whitaker, 2005). This report also confirmed that AFS1 transcript increased about four fold in peel tissue of apple fruit during the first 4 weeks of storage at 0.5 °C. In contrast, AFS1 mRNA declined over the initial 4 weeks of cold storage and was nearly undetectable by 8 weeks after fruit were treated at harvest with 1-MCP (Pechous and Whitaker, 2005). Recently, a study on targeted gene transcription profiling of defender against cell death (MD DAD1), defense no death (DND1) and lesion simulating disease resistance (MDLSD1) genes were reported and this suggested that there is an anti-apoptotic mechanism in scald development (Busatto et al., 2014).

Despite intensive investigation, the biochemical mechanism and genetic and molecular bases of apple scald development remain unknown. Intensive biochemical and physiological studies on disorders have mainly employed genomic and molecular approaches on limited set of genes and there is very little data available at the proteomic level to provide insight on the interaction of fruit ripening with the development of this physiological disorder.

Quantitative proteomics using non-gel based techniques has become a more popular approach that has shown the potential to expand our understanding of biology at proteomic level. Quantitative proteomic strategies that employ specific labeling techniques have been developed and applied to plant proteomic studies (Dunkley et al., 2006; Jones et al., 2006; Nelson et al., 2006; Owiti et al., 2011). A labeling strategy that involves the methylation of peptide amino groups *via* reductive amination with isotopically coded formaldehydes with ^{13}C has been developed (Boersema et al., 2008; Melanson et al., 2006). This stable isotope dimethyl labeling technique was reported as an efficient and cost-effective labeling strategy to detect differences in abundance ratios both global proteomic research and for protein modifications (Boersema et al., 2010).

To improve our fundamental understanding of scald development and its response to DPA and 1-MCP treatments, we conducted quantitative proteomic analyses to quantitatively investigate protein profiles of scald apple fruit tissues. We evaluated the proteins changes in response to DPA and1-MCP after 4 and 7 months of storage. The objectives of this study were to determine the protein profile changes in association with scald development in scald susceptible apple cultivars, identify protein changes in response to treatments that impart scald resistance with treatment of DPA and 1-MCP and reveal proteins that may be responsible for both scald development and resistance. The biological significance of protein changes is discussed within the context of apple fruit scald development and treatments.

2. Materials and methods

2.1. Apple fruit and treatments

Apple fruit (*Malus domestica*) "Cortland" and "Red Delicious" were harvested in 2013 from a commercial orchard in Berwick, NS, Canada. Fruit were harvested before the climacteric stage, with internal ethylene concentrations below 0.5 $\mu L\,L^{-1}$ (ca. one week before commercial harvest). Two biological replicates per cultivars of two tree blocks of more than 280 fruit were randomly harvested. After harvest, fruit were divided into three groups; control, DPA treatment and 1-MCP treatment. For DPA treatment, fruit were treated with Decco No Scald® DPA aerosol in a commercial storage room for overnight at the Scotian Gold Cooperative Limited (Coldbrook, Nova Scotia). 1-MCP (EthylBloc, 0.14%, Rohm and Haas Company, Philadelphia, PA) was applied at a concentration of 1.0 $\mu L\,L^{-1}$ to the 1-MCP group in a 342 L stainless steel chamber for 24 h. Both treatments were applied at 22 °C. Fruit were then stored under controlled atmosphere storage (CA) (3.0 kPa O_2 + 1.0 kPa CO_2) at 0–1 °C for 7 months. Fruit were removed and evaluated at 4 and 7 months storage. At each removal, fruit were held at 22 °C for 7 days. For each evaluation of samples, 12 fruit were used and evaluated.

2.2. Firmness, soluble solids and titratable acidity

Firmness was determined on both green and red sides of each individual fruit using a Magness-Taylor firmness tester (Model 30A; Ballauf Manufacturing Co., Laurel, Md.) with peel removed. Total soluble solids (TSS) and titratable acidity (TA) from 12 fruit were determined with a hand-held temperature-compensated refractometer (Atago Co., Tokyo) and measured using 0.1 mol L^{-1} NaOH employing a semi-automatic titrator (Multi-Dosimat E-415 titrator; Metrohm AG, Switzerland), respectively (Fan et al., 2011). Firmness, TSS and TA were evaluated at day 7 after removal.

2.3. Scald development

Scald development was evaluated immediately after removal from storage, and after an additional 7 days at 22 °C. Scald was rated using a scale of 1–5: 1 = no scald; 2 = 1–10%; 3 = 11–33%; 4 = 34–66%; and 5 = 67–100% (Watkins et al., 2000). For each evaluation, 12 apples were taken from each treatment group. Peels with 0.5 cm flesh tissue were pooled and frozen in liquid N_2, and stored at −86 °C for further analysis.

2.4. Protein extraction, quantification and digestion

Protein was extracted and purified from frozen ground fruit samples using a modified phenol extraction followed by ammonium acetate-methanol precipitation (Zheng et al., 2013). RC/DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to measur protein concentration and compensated the interfering compounds following the manufacturer's protocol. Bovine serum albumin was used as a protein standard.