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Transcriptional profiling of apple fruit in response to heat treatment: Involvement of a defense response during *Penicillium expansum* infection

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ABSTRACT

Heat treatment of harvested fruit has been demonstrated to be an effective and a safe approach for managing postharvest decay. In the present study, the effect of a hot water treatment (HT) (45 °C for 10 min) on the response of apple to blue mold infection was investigated. HT was applied to 'Ultima Gala' apples using 2 different methods. Wounded apples were: (1) inoculated with a *Penicillium* expansum spore suspension and then heat-treated after 1, 4 and 24 h (Inoc-HT); or (2) first heat-treated and then inoculated with a *P. expansum* spore suspension after 1, 4 and 24 h (HT-Inoc). All treated/inoculated apples were stored at 20 °C for 6 days. Significant reductions in fruit rot incidence, up to 100%, were observed using the Inoc-HT protocol at 4 and 24 h while a 30% reduction in blue mold incidence was found at 1 and 4 h using the HT-Inoc method. *In vitro* experiments showed no evident lethal effect of HT at 45 °C for 10 min on the germination of *P. expansum* conidia, indicating that this pathogen has a high heat tolerance. In order to investigate the molecular mechanisms involved in fruit response to heat treatment, an apple microarray was used to conduct a global transcriptional analysis of gene expression in apple at 0, 15, 30 min, 1, 4, 8 and 24 h after the heat treatment. The results provided evidence that at 1 and 4 h after heating, the HT apples had the highest number of differentially expressed genes. A significant upregulation of heat shock proteins, heat shock cognate protein, and heat shock transcription factor genes, involved in thermotolerance were observed. This indicates that the apple fruit respond to the heat treatment in a programmed manner and suggests that the genes responsible for thermotolerance may also be involved in the induced resistance response.

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1. Introduction

Apples are an economically important tree fruit crop, highly appreciated by consumers due to their sensory and nutritional characteristics (Giovanelli et al., 2014). Apples are consumed directly after harvest (fresh market) but are also stored for 6–12 months to provide continuous availability. The majority of the postharvest rots of apples that appear during storage are caused by fungal infections established on the fruit during harvest and processing in the packing house (Maxin et al., 2014). Restrictions on the use of fungicides established by regulatory agencies in the European Union and other countries, and consumer demand for fruit with residues much lower than regulatory levels, have increased the demand for organically grown fruit and stimulated research into alternative control measures. In some cases, the development of resistance may also impair the efficacy of fungicides used on key pathogens (Weber and Palm, 2010). Therefore, alternative strategies are needed to control the fungal postharvest disease. Among the physical treatments applied to fruit to control postharvest pathogens, hot water treatment of harvested fruit and vegetables has emerged as a safe and effective technique, having both a direct effect on pathogen propagules and the induction of defense responses in the harvested commodity (Fallik, 2004). Heat treatment has shown a promise in significantly reducing postharvest decay of apples (Fallik et al., 2001).

Neri et al. (2009) demonstrated the ability of an application of hot water treatment at 45 °C for 10 min to reduce natural infections of *Neofabraea alba* up to 90% in apple ('Pink Lady') after 135 d of storage at 2 °C. Maxin et al. (2012a) reported a significant reduction

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in the incidence of rots on naturally infected apples by incubating the fruit for 3 min at 50–54 °C (hot water dip) or 20–25 s at 55 °C (spray rinse). Rots were reduced by 80–100% and 60–80%, respectively. Maxin et al. (2012b), using fruit artificially infected with *Penicillium* expansum, reported a significant discrepancy between the temperatures lethal to spore suspensions *in vitro* and temperatures resulting in the inhibition of fruit rot (Maxin et al., 2012b). The authors reported an initial suppression of fruit rots with a treatment of 47 °C for 3 min while a direct inhibitory effect of heat on *P. expansum* in apples was evident only in pre-inoculated fruit subsequently exposed to 70 °C, which also scalded the fruit. Other effects resulting from heat treatment of fruit have been reported, including changes in the structure of epicuticular waxes resulting in the formation of deep cracks (Roy et al., 1994) which reduced the calcium uptake (Lurie et al., 1996), a decrease in the rate of softening of apples in both a non-storing, early, apple cultivar ('Anna'), and a long-storing, late, apple cultivar ('Granny Smith') after 4 days at 38 °C (Klein and Lurie, 1990), and the activation of multiple stress responses related to thermotolerance (Charng et al., 2006).

Sub-lethal heat treatments are known to have profound effects on the physiology of plant cells and tissues, including ripening fruit (Maxin et al., 2012b). Plants have evolved several mechanisms in order to withstand the stress of high temperatures (Wahid et al., 2007). These responses are triggered through a signaling cascade transmitted through the induction of Ca-dependent kinases that respond to stress and result in the activation of transcription factors and chaperone proteins. Among the induced proteins are several classes of heat shock proteins (HSPs) that range in molecular mass (Vierling, 1991; Gurley, 2000).

Global analysis of mRNA expression, using microarrays or next generation sequencing (NGS) has emerged as a valuable tool for providing a comprehensive picture of the transcriptomic response of plants to a wide range of biological processes such as disease resistance and susceptibility, environmental stress, and fruit development. This approach is particularly useful when the resulting transcripts can be aligned to a sequenced genome (Soria-Guerra et al., 2011; Vilanova et al., 2014). Understanding the molecular events associated with heat treatment of apple fruit could provide a valuable information for understanding the basis for optimal heat-treatment protocols. The present study utilized a 40,000 unigene apple microarray to conduct a comprehensive analysis of gene expression of the response of 'Ultima Gala' apple fruit to a hot water treatment. Samples were taken over a 24 h period after the heat treatment. The effect of HT on conidial germination of *P. expansum in vitro* and *in vivo* on artificially infected apples was also examined.

2. Materials and methods

2.1. Fruit

Apple (*Malus domestica* Borkh.) fruit from the cultivar 'Ultima Gala' were harvested from orchards located on the grounds of the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS Appalachian Fruit Research Station, Kearneysville, West Virginia, USA) and used immediately. The fruits were harvested on 9 August, 2012. Fruits were selected for uniform size and the absence of any physical injuries or apparent infection. Flesh firmness, soluble solids, and acidity were determined as quality parameters.

2.2. Quality parameters

Two measurements of flesh firmness, on opposite sides of the fruit, were made on each fruit using an 11-mm diameter plunger tip mounted on a drill press stand (Craftsman, Chicago, IL, USA). Total soluble solids content was assessed in juice using a refractometer (Atago, Tokyo, Japan), and titratable acidity by titration of 10 mL of juice with 0.1 N NaOH up to pH 8.2 and expressed as grams of malic acid per liter of juice. Data represent the mean and standard deviation of 15 fruit.

2.3. Fungal cultures

P. expansum strain (P100) was kindly provided by Dr. Wojciech Janisiewicz, also located at the USDA–ARS, Kearneysville, WV. Petri dishes containing potato dextrose agar (Difco, Sparks, MD, USA) were inoculated with the isolate and incubated at 25 °C for 7 days. A conidial suspension was prepared by adding 10 mL of sterile distillate water with 0.01% (w/v) Tween 80 to the Petri dish, gently scraping the surface of the colony with a sterile spatula, and then passing the wash water through 2 layers of cheesecloth to remove hyphal fragments. The concentration of the conidial suspension was adjusted to 10^4 conidia per milliliter using a hemocytometer for the *in vivo* assay and 10^6 conidia per milliliter for the culturable conidia assay.

2.4. P. expansum *culturable conidia assay*

Conidia viability was estimated as colony forming units (CFU) on PDA (Casals et al., 2010). Aliquots of 0.5 mL spore suspension (10^6 spores per milliliter) were added to 4.5 mL of water pre-warmed to 45, 50, 55 or 60° C. After 5 and 10 min of exposure, 0.5 mL of the warmed conidia suspension was immediately diluted 100-fold in cold water. Aliquots (0.1 mL) of treated *P. expansum* conidia suspensions were spread on Petri dishes and incubated for 3 days at 25 °C. A suspension of untreated conidia (10^3 conidia per milliliter) was used as a control. Approximately 100 spores of each pathogen per treatment were evaluated in the culturable conidia assay. Results were expressed as the number of CFU. The sample unit was comprised of 4 plates (replicates) and the experiment was conducted twice.

2.5. Inoculation of HT apple fruit with P. expansum

Apples were heat-treated and inoculated using 2 different protocols: (i) apples were artificially inoculated at time 0 and heat treated after 1, 4 and 24 h (Inoc-HT); (ii) apples were heat treated at time 0 and artificially inoculated after 1, 4 and 24 h (HT-Inoc). The heat-treatment of fruit consisted of immersing fruit in pre-warmed 45 °C water for 10 min. Control fruits were dipped for 10 min in water at 20 °C. Inoculation of apples was carried out in apples wounded with a nail (1 mm wide and 2 mm deep) and inoculated with 20 μL of a 10^4 mL^{-1} conidial suspension of *P. expansum*. Fruits were incubated at 20 °C for 6 days. Each biological replicate consisted of 5 fruit, and the experiment was repeated twice. The data was combined giving a total of $n = 10$. Data on pathogenicity were normalized using arcsine square root transformation and then analyzed for significant differences ($P < 0.05$) by analysis of variance (ANOVA) using an LSD test.

2.6. Fruit material for microarray and RT-qPCR analysis

'Ultima Gala' apples were harvested and immediately used. HT fruits were dipped in hot water at 45 °C for 10 min, control fruits were treated by dipping in RT water for the same exposure time. In order to obtain a general overview of gene expression in apple fruit in response to the heat treatment, samples of exocarp (skin) and mesocarp (fruit flesh) tissues were collected using a peeler. Each biological replicate consisted of a pool of skin and flesh tissues collected from 9 apple fruits. Control and heated tissues were