Effect of chitosan treatment on strawberry allergen-related gene expression during ripening stages

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Revised: 8 February 2017 / Accepted: 17 February 2017 / Published online: 24 February 2017
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Abstract The effectiveness of a chitosan coating treatment on the allergen-related genes expression of three strawberry cultivars (*Fragaria* × *ananassa* Duch., namely cvs. “Sabrina”, “Candonga” and “Jonica”) harvested at three ripening stages was evaluated. Fruits were treated with a 1% chitosan coating and stored at 20 °C for 48 h. Three genes, encoding Fra a1, Fra a3 and Fra a4 allergens, were evaluated by real-time qPCR. The transcript levels of several genes showed different patterns during ripening stages in strawberry cultivars but were up-regulated by chitosan treatment.

Keywords Chitosan · Strawberry · Allergen · Real-time PCR

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) belongs to the family *Rosaceae* and is one of the most appreciated and consumed berries. This fruit has remarkable nutritional quality due to the presence of micronutrient, antioxidant and phytochemical substances (Tulipani et al. 2011). Several studies demonstrated that the nutritional traits of

strawberries may be influenced by the genetic background of the cultivar or by pre- and post-harvest factors (Shin et al. 2008; Jin et al. 2011).

Strawberries, like other fruits from the *Rosaceae* family, contain allergenic compounds that are responsible for allergic reactions. The Allergome database (<http://www.allergome.org/>) currently lists three major strawberry allergens that were named Fra a 1, Fra a 3 and Fra a 4 by the WHO-IUIS subcommittee for allergen nomenclature. Fra a 1 is the first allergen described in the strawberry and belongs to the family 10 of plant pathogenesis-related proteins (PR-10); five potential isoforms (Fra a 1A–Fra a 1E) were identified with high sequence similarity and low amino acid variability as well as different immunological impacts (Hjerno et al. 2006; Musidlowska-Persson et al. 2007). Subsequently, two additional Fra a proteins, Fra a 2 and Fra a 3, were identified. These were homologous to proteins from the PR-10 group and showed 58 and 59% amino acid identity to Bet v 1 as well as 79 and 76% to Fra a 1E, respectively (Muñoz et al. 2010).

Fra a 3 belongs to the PR-14 protein family and is a non-specific lipid transfer protein type 1 (nsLTP1) with a molecular mass of approximately 9 kDa. Sequence alignment of Fra a 3 to PR-14 proteins from other *Rosaceae* fruits also showed the highest identity with the allergens of raspberry Rub I 3 (identity 78.6%), peach Pru p 3 (identity 73.6%) and apple Mal d 3 (identity 73.2%). Yubero-Serrano et al. (2003) characterized a strawberry Fxaltp gene (AJ315844.1) that contains two exons of 344 and 10 nt; the first 78 nt code for a putative signal peptide. This gene showed a tissue-dependent regulatory mechanism and responded differently to abiotic treatments, highlighting the importance of spatial expression studies to fully understand the role of this and other strawberry genes in defence (Yubero-Serrano et al. 2003). Zuidmeer et al. (2008)

Electronic supplementary material The online version of this article (doi:10.1007/s13197-017-2554-3) contains supplementary material, which is available to authorized users.

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demonstrated that Fra a 3 induced histamine release at a 100-fold higher concentration than peach LTP, although this does not seem to be clinically relevant because it has less allergenic potency than peach and apple LTP and is therefore an interesting tool for future immunotherapy.

Fra a 4 is a small (12–15 kDa) cytosolic protein belonging to the family of profilin proteins with a highly conserved molecular structure and 70–85% homology among different species. Profilins are found in all eukaryotic cells, and their allergenic potency has frequently been reported (Asero et al. 2003).

Chitosan is a natural biopolymer that is non-toxic for human health; can be applied as an antimicrobial agent against fungi, bacteria, and viruses; and can be used as an alternative compound to conventional fungicides due to its capacity to leave fruit surfaces free of residues. It has been used to control strawberry grey mould at the pre-harvest stage under controlled conditions in plastic tunnels or at the post-harvest stage by dipping strawberry fruit in the coating solution (Cao et al. 2011; Romanazzi et al. 2013; Feliziani et al. 2015; Petriccione et al. 2015a). Several studies have demonstrated that chitosan is a resistance inducer with dual action in plant protection; it inhibits the development of decay-causing fungi by the production of a film on treated surfaces and induces the resistance responses by the up-regulation of defence genes (El Gauth et al. 1992; Romanazzi et al. 2009; Landi et al. 2014).

The objective of this study was to investigate the effect of chitosan coating on the expression levels of allergen-encoding genes in three strawberry cultivars during different ripening stages.

Materials and methods

Fruit samples

Strawberries (cvs. Candonga, Jonica and Sabrina) were grown in controlled conditions in plastic tunnel greenhouses according to organic agriculture practices. Fruits were collected at three different ripening stages: green (approximately 7 days after blooming), pink (approximately 20 days after blooming), and red (approximately 30 days after blooming) and were immediately transported to the laboratory and screened for uniformity, appearance and the absence of physical defects or decay. The fruits at different ripening stages were then randomly distributed into two groups prior to treatment.

Strawberry fruits were dipped into the chitosan solution (1% w/w) for 60 s, prepared according to Petriccione et al. (2015b) to allow the chitosan to adhere to the whole fruit surface to create a uniform film. The samples were dried at room temperature, and three lots (300 g each) were

prepared in small PET boxes per cultivar per ripening stage. The same number of lots was prepared with control fruits dipped in distilled water. The fruits were stored in a controlled chamber at 20 °C and 95% relative humidity and were subsequently removed after 48 h according to Landi et al. (2014). The samples were stored at −80 °C until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from strawberry fruits, including achenes and the receptacle, using the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total RNA was treated with DNase (Invitrogen Life Technologies, Carlsbad, CA, USA), and the concentration of RNA was determined by UV/visible spectroscopy. Its structural integrity was checked on a non-denaturing agarose gel, followed by ethidium bromide staining. First-strand cDNA was synthesized from 3 µg of total RNA using oligo(dT)20 primers and the ThermoScript RT-PCR System (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations.

PCR primer design and amplification efficiency test

Gene-specific primers, encoding Fra a1, Fra a3 and Fra a4, were designed in our laboratory using Primer Expression software version 3 (Table 1). Actin (ACT) and 18S ribosomal RNA (18S-rRNA) as reference genes have been used previously (Landi et al. 2014) (Table 1). The amplification efficiency of each gene was determined using a pool representing all of the cDNA samples. First, all of the primers were examined by end-point PCR, all of the chosen were expressed, and specific amplification was confirmed by a single band of appropriate size in a 2% agarose gel after electrophoresis (data not shown). In a second step, the pool was used to generate a five-point standard curve based on a ten-fold dilution series. The amplification efficiency (E) and correlation coefficient (R^2) of the primers were calculated from the slope of the standard curve according to the equation: $E (\%) = (10^{(-1/slope)} - 1) \times 100$ (Radonic et al. 2004).

Real-time quantitative PCR analysis

Quantitative Real-time-PCR was performed using a CFX Connect Real-time PCR Detection System (Bio-Rad) to analyse the specific expression of each gene. cDNA was amplified in 96-well plates using the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), with 40 ng of cDNA and 300 nM specific sense and anti-sense primers in a final volume of 20 µl for each well. Each sample was analysed

Table 1 Descriptions of allergen-related gene and reference genes in *Fragaria × ananassa* and parameters derived from RT-qPCR analysis

Gene name (Gene symbol) Accession Number	Primer sequence (5'-3')	Amplicon length (bp)	Ta (°C)	PCR efficiency (%)	Regression Coefficient (R ²)	References
Allergen-related gene						
Fra a 1-A allergen (FRAA1A.1)	F: ATTGGAACTCGACAGGCAGC	104	59	102.2	0.994	This study
DQ385511.1	R: GGCATCTCCCTCGATCAAAC					
Lipid transfer protein (LPT46)	F: CTTTGGTCGCTCTCTTGTC	238	59	101.3	0.993	
AJ315844.1	R: GGGTTGAGTCCTTTGATGCC					This study
Profilin (<i>FRAA4</i>)	F: GAACCTGGAACACTTGCTCC	100	59	102.5	0.992	
DR027057	R: GCCCCTTCTTCCCTCTGATG					
Reference genes						
Actin (ACT)	F: CGAGGCTCAATCCAAAAGAG	159	55	98.5	0.995	Landi et al. (2014)
AB116565.1	R: GGGGCCTCAGTTAGGAGAAC					
18S Ribosomal RNA (18S-RNA)	F: CGCCAAGGAAGCTTGAATGAA	198	55	103.1	0.997	Landi et al. (2014)
AF163494.1	R: TTGCGTTCAAAGACTCGATG					

in triplicate. A sample without a template was used as the negative control, and a sample with non-retro-transcribed mRNA instead of template cDNA was used as the control for genomic DNA contamination. Thermal cycling was performed, starting with an initial step at 95 °C for 180 s, followed by 40 cycles of denaturation at 95 °C for 10 s and primer-dependent annealing (Table 1) for 30 s. To verify the specificity of the amplification, a melt-curve analysis was performed immediately after the amplification protocol. Relative fold changes in gene expression were calculated using the comparative $2^{-\Delta\Delta C_t}$ method and were normalized to the corresponding reference genes levels (Livak and Schmittgen 2001).

Statistical analysis

All of the experiments were replicated three times, and all data are expressed as the mean $\pm$ SD. Statistical analysis of data was performed by one-way ANOVA followed by the LSD post hoc test. Calculations were performed using SPSS v. 20.0.

Results and discussion

Strawberry allergen genes showed different expression patterns from the unripe to ripe fruit in a cultivar-dependent manner (Fig. S1). The relationship between allergens and the fruit-ripening process has also been studied in other fruit such as apple, peach and tomato (Brenna et al. 2004; Botton et al. 2009; Yang et al. 2012). Allergens have an important role as defence-associated proteins in response to biotic and abiotic stresses (Krebitz et al. 2003). Chitosan is

known to elicit plant defence responses by activating pathogenesis-related (PR) genes, and its use in organic agriculture has become a prospective alternative treatment to synthetic fungicides for control of post-harvest fruit and vegetable decay (Zhang et al. 2011). Previous studies have mainly focused on the effect of environmental conditions, harvest maturity, storage, and post-harvest treatment, as well as cultivar effects on the allergenic potential of different fruits, but few studies were carried out on strawberry (Yang et al. 2012; Muñoz et al. 2010). To our knowledge, this is the first study on the effect of chitosan coating on transcript levels of allergens in strawberry fruit during several ripening stages.

The gene encoding Fra a 1 is mainly expressed in unripe fruit (green), and the transcript levels decrease with the progression of the ripening process in a cultivar-dependent manner. After chitosan treatment, the Fra a 1 transcript levels in green strawberry fruits significantly increased by 1.42-fold, 2.43-fold, and 1.64-fold in “Sabrina”, “Candonga” and “Jonica”, respectively (Fig. 1a). Our results are in agreement with previous studies that assessed how Fra a 1 transcript levels decrease from the open flower stage to the ripe fruit stage by gene expression analysis performed using quantitative PCR (Muñoz et al. 2010). Fra a 1 is a PR-10 protein that acts in response to biotic and abiotic stress (Severo et al. 2015; van Loon e van Strien 1999). This protein has a large cavity in the hydrophobic pocket of its structure to improve interaction with ligands and may therefore facilitate the translocation of flavonoid molecules in ripening strawberry fruit (Hjerno et al. 2006; Muñoz et al. 2010). Although strawberry allergen Fra a 1 shows low sequence variability both within and between strawberry cultivars, different isoforms, due to differences

Fig. 1 Expression of FRAA1 (a), LTP46 (b) and profilin (c) genes, encoding Fra a 1, Fra a 3 and Fra a 4, in three strawberry cultivars (“Candonga”, “Jonica” and “Sabrina”) during different ripening stages (*green*, *pink* and *red*) in control and chitosan-coated fruits. Relative fold changes in gene expression were calculated using the comparative $2^{-\Delta\Delta C_t}$ method and were normalized to the corresponding reference genes levels (ACT and 18S-RNA). Values are means of three replicates $\pm$ SD

in post-transcriptional modifications, have been detected by 2-D electrophoreses (Alm et al. 2007; Musidlowska-Persson et al. 2007; Hjernø et al. 2006). Furthermore, chitosan treatment strongly increased the expression of 11 proteins of the pathogenesis-related protein-10 family, as well as their mRNA levels in *Vitis vinifera* cell suspensions (Ferri et al. 2009).

Fra a 3 transcript levels showed the same patterns during strawberry ripening stages in all analysed cultivars, with values that increased during colour development in the fruit that then decreased as the fruit approached the ripening stage (red fruit) (Fig. 1b). Chitosan treatment induced an increase in Fra a 3 transcript levels in all ripening stages, with the highest values in the intermediate stage of ripening (pink fruit), showing 6.4-fold, 6.2-fold and 10.5-fold increases at this stage for Fra a 3 in “Sabrina”, “Candonga” and “Jonica”, respectively.

Previous studies on the spatio-temporal expression pattern and structural features of the Fra a 3 gene in strawberry fruit development by northern analysis demonstrated a slight decrease in the expression of this gene during the intermediate and late ripening stages (Yubero-Serrano et al. 2003).

In contrast, the gene transcription rate for Fra a 4, a gene coding for a profilin, showed values that were not significantly different throughout the three stages of strawberry fruit development, with the highest values in chitosan-coated fruit (Fig. 1c). Previous studies demonstrated that the transcript levels of the two profilin genes (Pru p 4.01 and Pru p 4.02) in peach were almost constant (Botton et al. 2009) and that the different profilin genes (Mal d 4.01, Mal d 4.02, and Mal d 4.03) in apple slowly diminished during fruit ripening (Yang et al. 2012). The more stable patterns of profilin expression could be due to the putative actin-binding role reported for this protein, which is involved in important functions throughout fruit development such as cell growth and stability of the cytoskeleton (Schluter et al. 1997).

Conclusion

This study revealed the positive influence of chitosan coating on the regulation of gene expression of strawberry allergens during different ripening stages, providing additional information that could be helpful for the management of pre-harvest chitosan treatments to obtain fruits with low allergenic impact suitable for allergic individuals. Further studies using a proteomic approach are needed to evaluate how the differentially expressed allergen genes may be involved in the expression of their corresponding proteins in strawberry fruit during the ripening process.

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