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Genes differentially expressed in broccoli as an early and late response to wounding stress

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ABSTRACT

The plant wound-response is a complex process that generates changes in physiological, biochemical, and genetic mechanisms. The objective of the present study was to increase our understanding of the genetic wound-response of broccoli (*Brassica oleracea* L.) as an early (1 h) and late response (9 h) to two different wounding intensities (florets and chops) through transcriptome analysis by RNA-Seq. Chops generated the highest differential expression at both, early and late response; in the early response, genes that showed the highest upregulation were those involved in jasmonic acid biosynthesis and phenylpropanoid pathway, whereas in the late response those involved in amino acid and indolyl glucosinolate biosynthesis were upregulated. Likewise, in florets, only a few genes involved in the phenylpropanoid pathway were induced, mainly in the early response. The information generated will help to elucidate effective strategies leading to the enhancement of nutraceutical characteristics and shelf-life stability of fresh-cut broccoli products.

1. Introduction

Wounding by cutting is a common practice in the postharvest handling of fresh fruits and vegetables. This activity has increased in the last decades due to the growth of the ready to eat fresh-cut and frozen vegetables sectors (Cisneros-Zevallos et al., 2014). Wounding causes quality changes in the food product, as well as physiological, biochemical, and genetic alterations corresponding to defense mechanisms, such as those involved in signaling molecules and secondary metabolite production (Cisneros-Zevallos et al., 2014). Furthermore, wounding alters various aspects of the primary metabolism, like cellular respiration, photosynthesis, and sink/source relationships (Schwachtje and Baldwin, 2008; Jacobo-Velázquez et al., 2015). Also, it is known that the integration of different signals induced by wounding results in a complex cross-talk between the primary and secondary metabolism (Jacobo-Velázquez et al., 2015). Moreover, it has been established that the accumulation of secondary metabolites induced by wounding stress is partially due to the activation of the primary metabolism, because primary metabolites can work as defense compounds, signaling molecules, and as carbon source for the biosynthesis of secondary metabolites (Jacobo-Velázquez and Cisneros-Zevallos, 2002; Jacobo-Velázquez et al., 2015). Recently, it was reported that the application of wounding stress induces the accumulation of important bioactive compounds in broccoli (*Brassica oleracea* L.), such as phenolic compounds and glucosinolates (Villarreal-García et al., 2016; Torres-Contreras et al., 2017).

The dynamics and regulation of genes that contribute to wounding defense have not been deeply studied. In *Arabidopsis*, the highest gene expression response after wounding was observed few minutes (early response, between the first 30 and 90 min) and hours (late response, after 6 h and until 12 h) after wounding (Reymond et al., 2000; Cheong et al., 2002). Some genes involved in the phenylpropanoid pathway, and jasmonate and glucosinolate biosynthesis were reported to be wound-inducible and having different expression patterns after wounding (Cheong et al., 2002). The authors suggested that the time of induction of each gene reflects the position of the gene product in the response pathway. Thus, a transcriptome evaluation at an early and late response to wounding could provide the full panorama about the

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wound response that leads to the production of secondary metabolites in broccoli.

Therefore, the objective of the present study was to increase our understanding of the genetic wound-response of broccoli as an early (1 h) and late response (9 h) to two different wounding intensities (florets and chops) through transcriptome analysis by RNA-Seq. Increasing the scientific knowledge in this area is essential to design effective strategies leading to the enhancement of nutraceutical characteristics and shelf-life stability of fresh-cut broccoli products.

2. Materials and methods

2.1. Plant material, postharvest processing, and storage

Broccoli var. Heritage was harvested in Aguascalientes, in May 2016, and obtained in Monterrey (Nuevo León, México) from a local distributor. Broccoli heads were washed and disinfected with chlorinated water (0.02% of sodium hypochlorite solution, pH 6.5), and treated with two different wounding intensities: florets and chops. Whole broccoli heads were used as the control. Florets were obtained using a commercial straight-edged knife, whereas chops were done from broccoli florets using a commercial food processor (Waring Commercial, WFP11, Torrington, CT, USA). Samples were stored inside hermetic plastic containers with periodic ventilation (every 12 h) to avoid CO_2 accumulation higher than 0.5% (v/v). Two biological replicates were performed for each treatment. All samples (whole broccoli heads, florets, and chops) were stored at 20 °C in an incubator (VWR, Radnor, PA, USA) under dark conditions. Samples of each treatment were taken at 1 h (early response) and 9 h (late response) of storage time, immediately frozen with liquid nitrogen and stored at −80 °C until needed.

2.2. RNA extraction

Two independent RNA isolations of each sample were carried out. RNA extraction was performed following the hot borate method (Wan and Wilkins, 1994). RNA quality was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and RNA integrity was evaluated in 1% (w/v) agarose gel. Likewise, RNA integrity number (RIN) was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was treated with DNAse using RNAse Free DNAse (Qiagen, Hilden, NRW, Germany) and cleaned using the RNeasy Plant Mini kit (Qiagen, Hilden, NRW, Germany) following manufacturer's recommendations. Total RNA was evaluated with the following quality parameters: r26S/18S > 1, RIN > 8, OD 260/280 > 1.9 and OD 260/230 > 1.5.

2.3. Library preparation and RNA-Seq analysis

DNase treated RNA was used to prepare 12 separate Illumina sequencing libraries using TruSeq™ mRNA LT sample preparation kit (Illumina, San Diego, CA, USA), corresponding to two independent replicates of each wounding treatment (florets, chops), the control (broccoli head), and sampling times (1 h and 9 h). Before normalization and pooling, libraries were validated with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) using a DNA High Sensitivity chip. Libraries were sequenced (2 × 81 bp paired-end) at the facilities of Tecnologico de Monterrey (Monterrey, NL, Mexico) with the MiSeq Reagent kit v3 (150 cycles) using the MiSeq system (Illumina, San Diego, CA, USA).

2.4. Bioinformatic analysis

Sequenced reads from each sample were first assessed for quality using FastQC (Q ≥ 30; Andrews, 2017), adapters were trimmed with Trimmomatic (Bolger et al., 2014), and paired reads were mapped to the genome of *Brassica oleracea* (version 2.1, from http://plants.ensembl.org/Brassica_oleracea/Info/Index?db=core) using the default settings of Bowtie2 (Langmead and Salzberg, 2012). The concordantly paired reads that mapped to the genome were used for quantification of the gene level with HTSeq-count using the default settings (Anders et al., 2014). All these steps were performed with the Galaxy platform (Afgan et al., 2016). To determine differentially expressed genes (DEGs), the package DESeq2 version 1.14.1 (Love et al., 2014) was used in R version 3.3.2. DEGs were defined as those having a p-adjusted value ≤0.01, upregulated genes were defined as having a log2 fold change (log2FC) ≥ 1 and downregulated genes were defined as those having a log2FC ≤ −1. To visualize the overall effect of the treatments, principal component analysis (PCA) of counts normalized with a variance stabilizing transformation and a heat map showing gene expression according to treatment were constructed using DESeq2 and different packages available in R (R Development Core Team, 2015).

2.5. Quantitative RT-PCR

To confirm the differential expression of a set of genes a qRT-PCR approach was followed. The same RNA used for RNA-Seq analysis, as well as another independent RNA extraction of all samples, were used to synthesize cDNA with the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, Santa Clara, CA, USA) using random nonamers primers as described by the manufacturer's protocol. Quantification of transcripts, generated from cDNA, was performed in a Gene 3000 Rotor System (Corbett Life Science, San Francisco, CA, USA) with a 36-well rotor using the Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and primers for selected genes (Table 1). Conditions, procedures, and analysis of qRT-PCR data was performed as described by Salzman et al. (2005) using two biological replicates and three technical replicates for each gene validated (n = 6). Amplification specificity of each set of primers was determined by analysis of the cleavage curve and amplicon size on agarose gel electrophoresis, to ensure the absence of non-specific PCR products. Calibration curves of genes are shown in Supplementary Material (Fig. S1). Differential gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method following the protocol of Livak and Schmittgen (2001).

3. Results and discussion

3.1. Transcriptome gene expression analysis

To elucidate a comprehensive overview of postharvest wound-response in broccoli at the gene level, RNA-Seq libraries were designed including whole broccoli head (control), florets, and chops after 1 h (early response) and 9 h (late response) of storage at 20 °C. Sequencing of all treatments together produced a total of 28,495,468 high-quality reads. Paired-end reads from all treatments were mapped to the genome of *Brassica oleracea* version 2.1, obtaining a 79.81% of overall alignment rate.

A global view of the transcriptomes of broccoli under the different treatments is shown in Fig. 1. by means of a PCA (Fig. 1A) and a heat map (Fig. 1B). PCA showed that chops treatment grouped together and differed from the cluster generated by the florets treatment and the control (Fig. 1A). Also, the storage time explained some of the variances, except in the control where both storage times combine in the PC2-axis. In identified DEGs, no significant differences were found when comparing both storage time in control samples, thus from here and along the study whole broccoli heads at 1 h of storage was used as the control sample. Accordingly, chops exhibited the major difference in gene expression when comparing expression levels of the 50 genes with the highest variance in a heat map (Fig. 1B). The early response (1 h) in chops consisted of 3,007 DEGs (83% upregulated and 17% downregulated), while florets showed a lower response with 397 DEGs (94% upregulated and 6% downregulated). In chops, the late response