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Cloning and expression analysis of two putative papaya genes encoding polygalacturonase-inhibiting proteins

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ABSTRACT

Polygalacturonase-inhibiting proteins (PGIPs) are naturally occurring plant inhibitors that are able to retard the activity of fungal polygalacturonases (PGs) on pectin, and the manipulation of PGIP levels or the transfer of specific PGIP genes could reduce plant tissue decay. Because there is no information about PGIPs from papaya, even though this fruit is highly susceptible to fungal infection, two papaya PGIP genes were cloned, and their expression patterns were followed in different organs and tissues at different developmental stages. The *Cppgip4* and *Cppgip6* sequences share many features with other PGIPs. These genes were ubiquitously expressed in different organs and tissues and were more abundant in fruit pulp and peel. Both transcripts peaked when the fruit were still growing in size and then decreased at a late stage of development. A further reduction was observed during ripening, as both genes decreased significantly within 9 days after harvest. The down-regulation of PGIP genes during ripening was correlated to the decreased inhibitory activity of papaya protein extract against fungal pectinase from *Aspergillus niger*, and although the enzymatic assay did not provide the specific activity of each gene product, the finding suggests that protection against fungal PGs was impaired during ripening.

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1. Introduction

Diseases caused by fungal infection are a major cause of postharvest losses in papaya fruit (Dickman, 1994), potentially affecting more than 90% of production (Liberato and Costa, 1997; Tatagiba et al., 2002). Accordingly, strategies aimed at increasing the protection of fruit through induced or acquired resistance may be relevant for the post-harvest handling of papaya fruit. One of the first natural defenses of plant tissues is the pectin-rich amorphous structure of the plant cell wall, which is organized and arranged to provide a physical barrier while still enabling the growth of the cells. Because infections by pathogenic fungi progress through the release of cell wall-degrading enzymes (Huang et al., 2000), mainly pectinases, endogenous inhibitors present in plant tissues are important defensive elements for limiting disease (Cervone et al., 1989; Stotz et al., 1993, 1994).

Polygalacturonase-inhibiting proteins (PGIPs) are naturally occurring plant inhibitors that are able to retard the activity of fungal polygalacturonases (PGs) on the polygalacturonic acid chains of pectin (Cervone et al., 1987). PGIPs limit the destructive potential of exogenous PGs and allow the adequate accumulation of oligogalacturonides, which will trigger a cascade of defense responses (De Lorenzo et al., 2001; Di Matteo et al., 2006), such as the release of phytoalexins, ethylene, ROS, phenolic compounds and enzymes (Ridley et al., 2001; Federici et al., 2006; Shi et al., 2011).

PGIPs are members of an extracellular protein family with tandem repeats of leucine-rich sequences (LRR-leucine-rich repeats) (Jones and Jones, 1997), which are found in many pathogenesis-related proteins (Liu et al., 2013). Such repeats are typically composed of ten imperfect LRR motifs of approximately 24 amino acids each, and the combination of hydrophobic (leucine) and non-conserved residues confers binding specificity to fungal PGs (Di Matteo et al., 2003; D'ovidio et al., 2004).

The study of PGIPs in fruit has revealed that they are mainly expressed during development, when the fruit is immature (Johnston et al., 1993; Shivashankar et al., 2010). As development progresses, the level of PGIPs decrease, while at the same time, the susceptibility to fungal infection increases (Cantu et al., 2008). Therefore, PGIPs would provide better protection when the tissues

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are still growing, in contrast to the period of fruit ripening when the plant organ is about to release the seeds. Regarding the specificity of PGIPs to fungal PGs, the transfer of PGIP genes from resistant plants was effective at reducing the severity of fungal infection symptoms in the fruit of susceptible plants (Powell et al., 2000; De Lorenzo and Ferrari, 2002).

To date, there is no information about PGIPs from papaya, even though this fruit is highly susceptible to fungal infection. However, it has already been shown in other fruit that the manipulation of PGIP levels during fruit development or the transfer of PGIP genes specific to the pathogenic fungus of relevance could reduce fruit decay (Ferrari et al., 2003; Joubert et al., 2006; Landi et al., 2014). Therefore, to provide baseline information on PGIPs from papaya, we aimed to clone putative genes of papaya PGIPs and examine the expression pattern of those genes in different organs and tissues of the plant at different developmental stages, mainly during fruit development and ripening.

2. Material and methods

2.1. Plant material

Papaya (*Carica papaya* L.) fruit cv. 'Golden' were harvested in January 2010 and January 2011 from a commercial farm in the municipality of Aracruz/ES, Brazil, latitude 19°24′S and longitude 40°04″W. Physiologically immature fruit was harvested at 30, 60, 90 and 110 days after anthesis (DAA) according to a previous report (Silva et al., 2010). Samples of physiologically mature fruit were harvested at color break to 1/4 yellow at approximately 140 DAA. Immature fruit and their seeds were immediately frozen in liquid nitrogen; mature fruit was left to ripen spontaneously in a 240-dm^3 chamber with a controlled temperature and humidity (25 °C ± 0.1 °C and 95%, respectively). At each day of ripening, the pulp and peel were removed and immediately frozen in liquid nitrogen. At least five fruits were collected at each sampling point during development or ripening. All samples were stored at −80 °C.

For other tissues (stems, roots and leaves), two samples were collected in consecutive years (2012 and 2013), at the same time of year, between the months of January and March. Samples from plants at three and six months of age were collected. At least five representative plants of each phase were collected at each sampling, and the organs used in the analysis were frozen and stored as described above.

2.2. Respiration, ethylene production and pulp firmness

The respiration and endogenous ethylene production of fruit were measured on a daily basis, as previously described (Fabi et al., 2007). At least five fruits were individually placed in airtight jars and left at 25 °C for 1 h. After this time, samples of 10 mL for ethylene analysis and 1 mL for CO_2 analysis were collected, and the composition of gases was determined by gas chromatography.

The firmness of the same five fruits used for the measurement of respiration and ethylene production was analyzed using a handheld penetrometer (Effegi FT 327) with an 8-mm plunger tip and expressed as the puncture force in Newtons.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was isolated from pulp, peel, roots, stems, seeds and leaves using Concert Plant RNA Reagent (Life Technologies) according to the manufacturer's instructions. Quantitation and cDNA synthesis were performed as described (Fabi et al., 2010). Briefly, spectrophotometrically quantified RNA (quadruplicate) was treated with DNase RNase-free (NucleoSpin®–Macherey Nagel©), and first-strand cDNA was synthesized with random primers using 1 μg total RNA DNA-free and the ImProm-II Reverse Transcription System (Promega).

2.4. Papaya PGIP gene identification and multiple protein sequence alignment

To search for papaya PGIPs, known PGIP genes from diverse plants were aligned individually against a papaya whole-genome shotgun (WGS) database using the BLASTN tool (score ≥100 and e-value ≤ 1e-30 as cut-off values). Genes from *Malus domestica* (AY347793.1), *Vitis vinifera* (AF499451), *Actinidia deliciosa* (Z49063.1), *Cucumis melo* (AY288911), *Pyrus communis* (L09264), *Prunus persica* (EF409977), *Prunus armeniaca* (AF020785), *Solanum lycopersicum* (L26529) and *Rubus idaeus* (AJ620355) were used for comparison. After primer design, putative coding regions were PCR-amplified using high-fidelity KOD Hot Start DNA Polymerase (Novagen®), cloned and sequenced four times. After cloning the cDNA of papaya PGIPs and deducing their amino acid sequences, a multiple sequence alignment was performed with the following PGIP protein sequences from dicots: *M. domestica* (AY347793.1), *V. vinifera* (AF499451), *A. deliciosa* (Z49063.1), *C. melo* (AY288911), *P. communis* (L09264), *P. persica* (EF409977), *P. armeniaca* (AF020785), *S. lycopersicum* (L26529), *R. idaeus* (AJ620355), *A. thaliana* (Q9M5J8 and AAM65836), *F. ananassa* (A7Y2Y8), *B. napus* (Q8L579), *C. hystrix* (BAB82980 and O80421), *T. cacao* (XP00702894), *P. vulgaris* (P58822 and P35334), *G. max* (Q0WX05 and Q0WX04) and *V. corymbosum* (B6V8Z6). A phylogram tree was constructed using neighbor-joining analysis of a distance matrix generated with ClustalW2. Homology-based structural modeling of the papaya PGIP domains was performed using the SWISS-MODEL software (http://www.expasy.org) (Schwede et al., 2003) with a first-approach method based on the *P. vulgaris* PGIP template (Di Matteo et al., 2003).

2.5. Quantitative RT-PCR expression profiling

Papaya PGIP gene sequences were evaluated following 'Minimum Information for Publication of Quantitative Real-Time PCR Experiments – MIQE' (Bustin et al., 2009); primers were designed according to a previous report (Fabi et al., 2012). The primers were as follows: 5′-CCGGATCGACCAGCTAAATA-3′ sense and 5′-CCGGTGAGGTTTGTGAGTTT-3′ antisense for *Cppgip4*; 5′-CAGTTCCCGACTTTCTCAGC-3′ sense and 5′-CGGATGGATG-TAAGGTTTGG-3′ antisense for *Cppgip6*. As internal controls (reference genes) for relative expression, we used translation elongation factor (*tef*), with the primers 5′-GTTAAGAACGTTGCCGT-GAAG-3′ sense and 5′-ATGTGAAGTTGGCTGCTTCCT-3′ antisense, and the ubiquitin (*ubq*) gene, with primers 5′-ACTCACCGGCAA-GACCAT-3′ sense and 5′-GTGGAGAGTCGATTCCTTTTG-3′ antisense. The transcript levels of the reference genes were not influenced by the developmental stage, as observed by geNorm software analyses, and the geometrical means of the Ct values were calculated for the analysis of relative expression.

Real-time RT-PCR was performed in a final reaction mixture of 10 μL containing 2 μL of cDNA, 0.2 μL of 10 μM primer and 5.0 μL of Platinum® SYBR® Green qPCR Supermix-UDG with ROX. Real-time PCR was performed in a Rotor-Gene 3000 (Corbett research) according to the following program: 2 min at 50 °C, 5 min at 95 °C and 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. No-template controls (NTCs) and melting curves of the amplicons were analyzed for all experiments. The expression values are given as the mean of the normalized expression values of quadruplicates calculated according to the equipment software, and quantification was performed using the relative standard curve method (Pfaffl, 2001).

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