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Induction of the expression of defence genes in *Carica papaya* fruit by methyl jasmonate and low temperature treatments

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Abstract The defence mechanisms that are activated by methyl jasmonate (MJ) in fruits are not well understood. In this work, we studied the expression of defence genes in papaya fruit that are induced by the exposure to MJ and/or low temperatures. The papaya fruits 'Maradol' were randomly divided into two groups: one group was the untreated control and the other was treated with 10^{-4} M of MJ. Half of the fruits from each of the two groups were stored after treatment for 5 days at 5ºC and 2 days at 20ºC. We studied the expression levels of the *pdf1.1* and *pdf1.2* genes by amplification from expression libraries created from the pulp and skin tissues of the papaya fruit. As a reference, the mRNA level of the 18s ribosomal gene was used. In the skin tissue, the expression levels of the *pdf1.1* and *pdf1.2* genes were higher immediately after MJ treatment compared to the control. Furthermore, the expression of *pdf1.2* remained high after MJ treatment and subsequent storage compared to the control. It was therefore concluded that the activation of the *pdf1.1* and *pdf1.2* genes forms part of the molecular defence mechanism in fruits that is activated by exposure to MJ. To our knowledge, this is the first study that analyzes the gene expression in papaya fruit that is induced by the exogenous application of methyl jasmonate and cold treatment.

Keywords: cold treatment, defence proteins, genetic expression, jasmonic acid methyl ester, papaya fruit

INTRODUCTION

The responses of plants to biotic and abiotic stresses are induced locally and systematically by molecules known as jasmonates (Avanci et al. 2010). These molecules are synthesized by the octadecanoic acid metabolic pathway (Creelman and Mullet, 1995; Beale and Ward, 1998). Methyl jasmonate (MJ), which is the methyl ester derivative of jasmonic acid, is a volatile molecule that regulates various developmental processes and defence responses in plants (Seo et al. 2001; Nahar et al. 2011). MJ is a ubiquitous compound in fruits and vegetables and its exogenous application can induce the synthesis of proteins known as jasmonate-induced proteins (Afroz et al. 2010). In addition, jasmonates induce the activation of the plant defence system in response to pathogen attack (Reymond and Farner, 1998; Sabater-Jara et al. 2011), to wounds that are mechanically caused (Maciel et al. 2011) and to insect herbivores (Wünsche et al. 2011). Furthermore, MJ is involved in the signal transduction pathways that are initiated in response to various stresses and it has been shown that MJ affects the gene expression in tissues exposed to vapours or solutions at concentrations between 10^{-4} and 10^{-5} M (Reinbothe et al. 1994).

Plant defensins are genes that encode small proteins with a three-dimensional structure stabilized by eight disulfide bonds (Campopiano et al. 2004). Their name, defensins, has been adopted because these are structurally related to the defensins that have been identified in other organisms, including humans (Wong et al. 2007). These proteins have been recorded in many plant species (Gachomo et al. 2012). In fact, in the *Arabidopsis thaliana* genome, approximately 13 defensins and 317 defensin-like genes have been identified (Graham et al. 2008). The importance of these proteins arises from their potent inhibitory activity to many fungi species without exhibiting any known toxicity to the plant cells (Thomma et al. 2002). The *pdf1.2* defensin gene is induced by pathogen attack in *Arabidopsis*, whereas the *pdf1.1* gene is preferentially expressed in the *Arabidopsis* fruit (Penninckx et al. 1996).

It has been reported that the induction of the *pdf1.2* gene after exposure to MJ is blocked in the *ethylene-insensitive 2* (*ein2*) mutant of *Arabidopsis thaliana*, which suggests that the ethylene signal transduction pathway is involved in the induction of the defence system by MJ in plants (Penninckx et al. 1998; Yu et al. 2011). Accordingly, it was found that the induction of the *pdf1.2* gene in the leaves of *Arabidopsis thaliana* requires the presence of both MJ and ethylene (Penninckx et al. 1998). In addition, it had been reported that the induced expression of defence genes in the banana fruit by MJ seems to require the presence of ethylene, which suggests that the mechanisms activating the defence system in the fruit are similar to those of the leaf tissue (Tang et al. 2010).

In addition, it was found that the *pdf1.1* gene is induced in plant species that live in very low temperature environments, which implies that the induction of this gene is associated with the response of plants to low temperature stress (Archambault and Strömvik, 2011).

Chilling injury (CI) is a biochemical and physiological disorder that is prevalent in tropical and subtropical fruits that are subjected to temperatures between one and 15ºC, which does not freeze the tissue (Wang and Buta, 1994). In papaya, the exposure of tissues to temperatures below 10ºC can cause CI depending on the storage conditions and cultivar (Chen and Paull, 1986; Sevillano et al. 2009). Among several approaches that have been undertaken to reduce CI, MJ is a promising commercial alternative for the maintenance of optimal fruit quality during prolonged storage periods at low temperatures. Indeed, it was reported that the treatment of papaya fruit with MJ can reduce the CI physiological disorder (González-Aguilar et al. 2003). A reduction in CI has also been reported with the application of MJ to tomatoes (Fung et al. 2006), pomegranates (Sayyari et al. 2011), peaches (Jin et al. 2009a) and Japanese loquats (Cao et al. 2009). Similarly, an increase in the synthesis of MJ was discovered in mango and banana fruits exposed to temperatures below 12ºC, which suggests that jasmonate is part of the response of the fruit to low temperature stress (Kondo et al. 2005).

Furthermore, it has also been reported that the induction in the synthesis of MJ is part of the tolerance's mechanism of apple fruit (Yoshikawa et al. 2007) and pomegranate (Zolfagharinasab and Hadian, 2007) to the low temperatures effects.

In addition to reducing CI symptoms, MJ has been reported to decrease fungal infection in various fruits, such as Chinese strawberries (Wang et al. 2009), tomatoes (Tzortzakis, 2007), peaches (Jin et al. 2009b), Japanese loquats (Cao et al. 2009), bananas (Zhu and Ma, 2007) and strawberries (Zhang et al. 2006). It has been found that this reduction in fungal infections in fruits by MJ involves the induction of several enzymes that play a role in the defence against pathogen attack. Indeed, the induction of various isoenzymes of chitinase and β-1,3-glucanase has been found in bananas (Zhu and Ma, 2007), strawberries (Zhang et al. 2006), tomatoes and bell peppers (Wang et al. 2005). Moreover, the induction of the expression of pathogenesis-related protein 1 and chitinase in bananas has been observed in response to treatment with MJ (Tang et al. 2010). However, the mechanism by which MJ reduces the postharvest fungal rot of fruits has not yet been fully elucidated.

The purpose of this study was to improve the understanding of the fruit defence system that is induced in response to low temperatures and MJ treatments. Therefore, the induction of the *pdf1.1* and *pdf1.2* genes by MJ treatment was studied in papaya fruits that were exposed to low temperatures.

MATERIALS AND METHODS

Plant materials

The papaya fruits 'Maradol' were collected from the Coliman group of Hermosillo, Mexico and transported immediately to the facilities of the Centro de Investigación en Alimentación y Desarrollo at Hermosillo Sonora to be selected. Those fruits that had any physical injuries or slight signs of deterioration were discarded and the selection of the remaining fruits was based on colour, size and shape. The fruits were then randomly divided into two groups of 10 fruits each: an untreated control group and a group that was treated with 10^{-4} M of MJ. The application of MJ was carried out by immersion in an aqueous solution of 10^{-4} M of MJ for 3 min. Tween 20 at a concentration of 0.01% was added to the aqueous MJ solution to facilitate the dissolution of MJ and thus enhance the contact of MJ with the fruit. An aqueous solution of 0.01% Tween 20 without MJ was used for the treatment of the control fruits. After soaking, the fruits were left to dry at room temperature for 4 hrs. A section of the fruit pericarp from each of five treated and five control fruits was then removed and the pulp tissue was separated from the skin tissue. Both the pulp and peel tissues were then cut into 1 x 2 cm slices, immediately frozen in liquid nitrogen and stored in individual bags at -80ºC. The samples were labelled as follows: unstored control pulp (UCP), unstored control skin (UCS), unstored MJ-treated pulp (UTP), and unstored MJ-treated skin (UTS). The remaining fruits in the MJ-treated and control groups were stored for 5 days at 5ºC and then for 2 days at 20ºC. After this time, the samples were prepared in the same manner as the unstored samples and stored separately at -80ºC; these were labelled as follows: cold stored control pulp (SCP), cold stored control skin (SCS), cold stored MJ-treated pulp (STP) and cold stored MJ-treated skin (STS).

Total RNA extraction

The total RNA from each of the pulp and skin samples of the papaya tissues was extracted according to the hot phenol micro scale technique described by Scott (1995), which includes the use of a 4 M lithium chloride solution to precipitate the RNA. The ribonucleic acid integrity was evaluated on a 0.8% agarose gel containing ethidium bromide and the presence of the 18s and 28s bands of the ribosomal RNA was used as the criteria.

Synthesis of complementary DNA (cDNA)

The synthesis of the complementary DNA from the total RNA of the papaya samples was performed following the manufacturer's instructions (cDNA Library Construction Kit, Clontech Laboratories, Inc. Mountain View, CA, USA). The subsequent PCR was carried out with 21 cycles of the Gene Amp 480 program with the following set of protocols for each cycle: 95ºC for 1 min, 95ºC for 15 sec, and 68ºC for 6 min. The cDNA was then treated with proteinase K and digested with the *Sfi*I restriction enzyme. Once digested, the cDNA was fragmented by a chromatography column (CHROMA SPIN 400). The aliquots obtained from the column separation were ligated into the *Sfi*I-digested vector pDNR-LIB. The concentration of cDNA obtained was quantified in a Nano-Drop 1000 (Nano Drop Inc. Technologies, USA) and 50 ng of cDNA was used for the PCR reactions.

PCR analysis of *pdf1.1* and *pdf1.2* genes

The cDNA libraries obtained from all the samples were used in PCR assays with specific primers for the genes encoding the 18s ribosomal RNA (18s RNA), the plant defensin *pdf1.1* and the plant defensin *pdf1.2*. The oligonucleotide sequences utilized in the assays are shown in Table 1. The products obtained from the PCR amplification (1 µg/µL) were separated by 1% agarose gel electrophoresis with ethidium bromide and photographed by the Gel Logic 100 Imaging System Performance using an ultraviolet transilluminator (KODAK).

Quantification of the bands resolved on the agarose gel electrophoresis

The analysis of the intensity of the DNA fragments separated by agarose gel electrophoresis was performed using the program Image J 1.42q (http://rsb.info.nih.gov/ij). In this software, the intensity value of the band (mean gray value) is related to the average of the gray colour readings inside the selected area, which is the sum of all gray or luminosity values in all the pixels of the selected area.

Areas of 0.018, 0.016 and 230 mm^2 were used to analyze all the amplified fragments of *pfd1.1*, *pdf1.2* and 18s ribosomal RNA, respectively; these same areas were used in the analysis of each sample.

Table 1. Sequence of specific primers and expected size of the amplified fragments for each of the genes used in the analysis of the MJ-treated or untreated papaya fruit tissues.

Gene Analyzed	Primer Sequence	Amplified Fragment (bp)	Reference
RNA ribosomal 18s (18s RNA)	F: 5'-CCTGCGGCTTAATTGACTC-3' R: 5'-GTTAGCAGGCTGAGGTCTCG-3'	174	Grando et al. 2005
Plant Defensin (PDF1.2)	F: 5'-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3' R: 5'-AATCCATGGAATACACACGATTTAGCACC-3'	350	Penninckx et al. 1996
Plant Defensin (PDF1.1)	F: 5'-GAGAGAAAGCTTGTTGTGCGAGAGGCCAAGTGGG-3' R: 5'-GAGAGAGGATCCTGCAAGATCCATGTCGTGCTTTC-3'	127	Penninckx et al. 1996

F: Forward, R: Reverse.

RESULTS

Total RNA and cDNA from papaya pulp and skin tissues

Figure 1a illustrates the agarose gel of the total RNA extracted from the tissue samples; as shown, the presence of the 18s and 28s bands can be clearly observed, which strongly supports the fact that the RNA utilized for the cDNA synthesis was not degraded. In addition, Figure 1b shows the agarose gel of the cDNA synthesized from the total RNA in all samples along with the molecular weight markers of the lambda genomic DNA that was digested with *EcoRI* and *Hind*III. The cDNA used to create the expression libraries was chosen to have a molecular weight between 500 and 3200 bp.

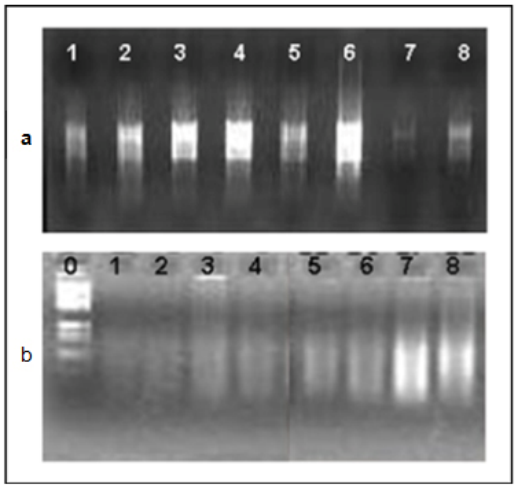

Fig. 1 Agarose gel of total RNA (a) and cDNA synthesized from total RNA (b). Lane 0: Lambda molecular weight marker digested with *EcoRI* and *Hind*III; Lane 1: UTP = unstored MJ-treated pulp tissue; Lane 2: UCP = unstored pulp tissue (control); Lane 3: UTS = unstored MJ-treated skin tissue; Lane 4: UCS = unstored skin tissue (control); Lane 5: STP = cold stored MJ-treated pulp tissue; Lane 6: SCP = cold stored pulp tissue (control); Lane 7: STS = cold stored MJ-treated skin tissue; Lane 8: SCS = cold stored skin tissue (control). The presence of the 18S and 28S bands in panel a shows that the RNA extracted is of good quality. In panel b, the cDNAs that were amplified to create the expression libraries from the control and MJ-treated samples are shown. The cDNAs chosen were between 500 and 3200 bp in size.

PCR assay of 18s rRNA internal control gene

The internal amplification control that was used to analyze the quality of the cDNA obtained from the different papaya samples was the 18s ribosomal RNA (18s rRNA). The results of the amplification of the 18s rRNA are shown in Figure 2. In the gel, a band of approximately 174 bp can be clearly observed in the skin and pulp tissues of the control and MJ-treated samples that were not subjected to cold temperature storage. Furthermore, the results of the densitometric analysis of the bands are shown, which clearly suggests that the amount of RNA is the same in all the samples analyzed.

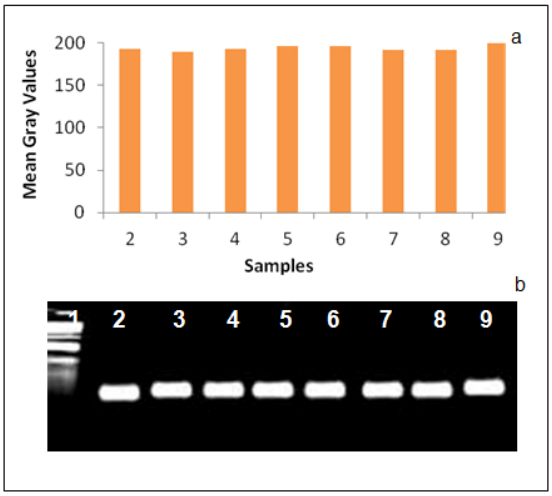

Fig. 2 Densitometry analysis (a) and photograph (b) of the agarose gel of the amplified 18s rRNA band. Lane 1: molecular weight marker, 1 kb + DNA ladder; Lane 2: UTP = unstored MJ-treated pulp tissue; Lane 3: UCP = unstored pulp tissue (control); Lane 4: UTS = unstored MJ-treated skin tissue; Lane 5: UCS = unstored skin tissue (control); Lane 6: STP = cold stored MJ-treated pulp tissue; Lane 7: SCP = cold stored pulp tissue (control); Lane 8: STS = cold stored MJ-treated skin tissue; Lane 9: SCS = cold stored skin tissue (control). The densitometric analysis of the 174 bp amplified band from the 18S ribosomal RNA clearly showed that the same amount of RNA was present in the different samples that were utilized to create the expression libraries. In the graph of the densitometric analysis, the Y axis represents the mean gray value.

PCR assay of the *pdf1.1* and *pdf1.2* genes

In Figure 3a, the amplification of the *pdf1.1* gene in the tissues of the papaya fruit after the treatment with MJ (top) and the subsequent cold storage (bottom) is presented. The expression level of this gene in the skin tissues immediately after treatment with MJ (UTS) was higher than its expression level in the skin tissue of the control fruits (UCS). After storage of the fruit in cold temperatures, there were no differences in the expression of *pdf1.1* between the treatments or between the tissues analyzed, which indicates that treatment with MJ induces a transient expression of this gene immediately after treatment and therefore this gene exhibits an early response in the papaya skin tissue to exogenous MJ. In addition, a slight increase in the expression of this gene was observed in the MJ-treated skin tissue that was stored in cold temperature (STS) compared to the untreated and unstored skin tissue (UCS) control.

The expression level of the *pdf1.2* gene (Figure 3b) immediately after treatment (top) in the pulp tissue was higher in the control fruits (UCP) compared to the MJ-treated fruits (UTP). Furthermore, this behaviour profile is maintained after storage in a cold temperature. In contrast, the expression level of this gene in MJ-treated skin tissues (UTS) was higher than in control skin tissues (UCS) immediately after MJ treatment. Moreover, the *pdf1.2* gene levels in the skin tissues after cold storage (bottom) were higher in MJ-treated tissues (STS) than in control tissues (SCS). After cold storage, the gene expression levels of the *pdf1.2* gene were reduced in the skin tissues obtained from both treatment groups (STS and SCS) compared to the expression levels in the corresponding skin tissue measured immediately after treatment (UTS and UCS).

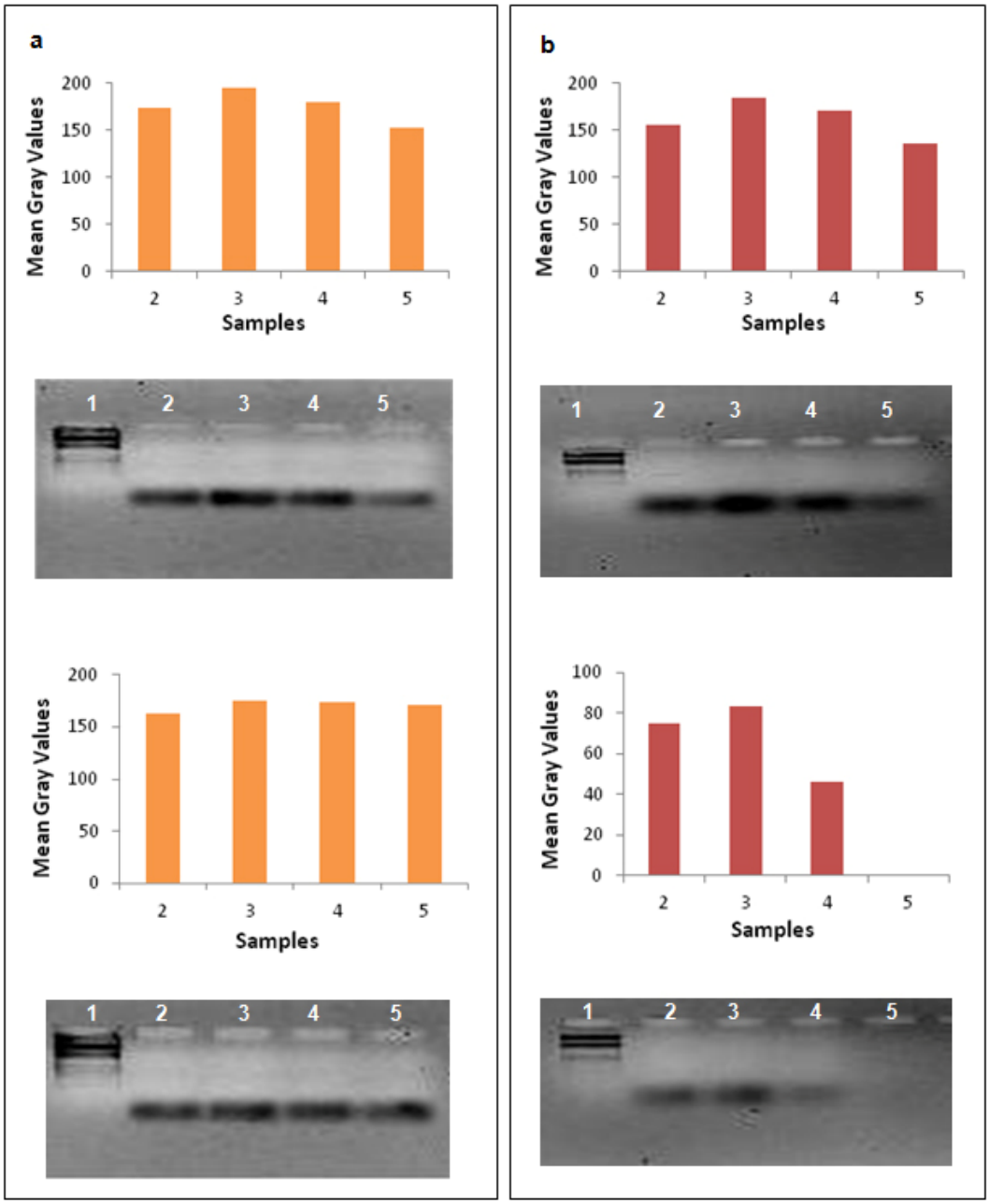

Fig. 3 Graph of densitometric analysis and photograph of the agarose gel with the bands associated with the *pdf1.1* (a) and *pdf1.2* (b) genes. In the top panels of a and b: Lane 1: molecular weight marker 1 kb plus DNA ladder; Lane 2: UTP = unstored MJ-treated pulp tissue; Lane 3: UCP = unstored pulp tissue (control); Lane 4: UTS = unstored MJ-treated skin tissue; Lane 5: UCS = unstored skin tissue (control). In the bottom panels of a and b: Lane 1: molecular weight marker 1 kb plus DNA ladder; Lane 2: STP = cold stored MJ-treated pulp tissue; Lane 3: SCP = cold stored pulp tissue (control); Lane 4: STS = cold stored MJ-treated skin tissue; Lane 5: SCS = cold stored skin tissue (control). The expression levels of *pdf1.1* and *pdf1.2* genes in the skin tissues were higher after treatment with MJ compared to the control (top panels of a and b: Lanes 4 and 5). Cold storage did not affect the expression levels of the *pdf1.1* gene (bottom panel of a); however, the expression levels of *pdf1.2* after cold storage were higher in the MJ-treated skin tissues compared to the control (bottom of panel b: Lanes 4 and 5). In addition, the *pdf1.2* gene showed a reduced expression level in all treatments after cold storage compared to the corresponding unstored samples (bottom panel of b). In the densitometric analysis, the Y axis represents the mean gray value.

DISCUSSION

The constitutive gene *18s rRNA*, similar to the elongation factor gene *1-α(ef1α)* and *β-tubulin*, is one of the genes in plants that exhibits the most stable behaviour during the response to biotic and abiotic stress. Therefore, these genes can be used as suitable internal controls in PCR amplification and real-time PCR experiments to quantify the effects of different stress conditions on gene induction (Nicot et al. 2005; Sato et al. 2005). The densitometric analysis results shown in Figure 2 clearly demonstrate that all the samples that were collected have the same amount of cDNA, which strongly supports the validity of the results obtained in the gene expression analysis.

The defence proteins PDF1.1 and PDF1.2 have been linked to the action of jasmonates in plants that are exposed to low temperature stress (Penninckx et al. 1998). In this study, the expression of both of these genes in the pulp and peel tissues of papaya fruits treated with MJ and stored in cold temperatures was studied. The present work found an increase in the expression of both the *pdf1.1* and *pdf1.2* genes in response to MJ treatment in the skin tissue of the papaya fruit. A slight increase was observed in the expression of the *pdf1.1* gene in the skin tissue of the control fruit after storage (SCS) compared to the unstored control skin tissue (UCS) sample; this increase might be because this gene has been shown to be induced in response to low temperature stress (Archambault and Strömvik, 2011). In addition, it has been reported that the *pdf1.1* gene is differentially expressed in the *Arabidopsis* fruit (Penninckx et al. 1996), which may explain the higher expression levels of this gene in the unstored control pulp tissues (UCP) compared to the unstored MJ-treated pulp samples (UTP). However, papaya fruit tissues might not respond in the same way to MJ treatment as *Arabidopsis* fruits.

It has been reported that the *pdf1.2* gene is induced in *Arabidopsis* leaves in response to the stress response that is induced by pathogens and to the exogenous application of MJ (Penninckx et al. 1996). This work found an induction of this gene in the skin of unstored and MJ-treated papaya fruit (UTS) compared to the control samples (UCS), which is in agreement with previously published results. The *pdf1.2* gene of *Arabidopsis* encodes a defensin gene that is commonly used as a marker for the characterization of the jasmonate-dependent defence response (Brown et al. 2003), and its expression has been observed in *Arabidopsis* leaves as a response to the presence of pathogens and MJ (Penninckx et al. 1996; Penninckx et al. 1998). The findings of this study suggest that MJ may induce the expression of this gene as a defence mechanism in the skin of papaya fruits in a similar way as in the leaves, which suggests the presence of analogous defence mechanisms in the vegetative and fruit tissues of plants.

Plants must withstand continuous exposure to biotic and abiotic stresses in their natural environment. Thus, to respond and adjust to these environmental stresses, plants have evolved mechanisms to perceive external signals that result in the repression or induction of specific genes (Pieterse et al. 2009). The perception of stress signals includes the activation or production of one or more secondary signalling molecules, such as salicylic acid, ethylene or jasmonates. The jasmonates are a group of signalling molecules that are derived from lipids, including jasmonic acid (Wasternack, 2007). These signalling molecules are involved in the defence response of plants. The exogenous application of this hormone, MJ, or its analogues results in the induction of a number of different proteins that are related to the defence system of plants, such as thionins in barley (Andresen et al. 1992) and *Arabidopsis* plants (Epple et al. 1995), protease inhibitors in tomatoes (Farmer and Ryan, 1992) and defence proteins (Penninckx et al. 1996; Penninckx et al. 1998).

The induction of the *pdf1.1* and *pdf1.2* genes in the peel of the papaya fruit treated with MJ and stored in a cold temperature presumably indicates the activation of the defence mechanism of this tissue. The participation of the *pdf1.2* gene in the plant response to biotic stress is well-documented. For example, the exposure of A*rabidopsis* to the fungus *Piriformospora indica* showed high induction levels of the *pdf1.2* gene (Stein et al. 2008). The defence response of this gene has also been studied in different transgenic tissues. Transgenic plants that over express the jasmonic acid carboxyl methyltransferase gene, which encodes S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase, an enzyme that catalyzes the formation of methyl jasmonate from jasmonic acid, have been found to exhibit constitutive expression of jasmonate-responsive genes, including the vegetative storage protein and the PDF1.2 defence protein. In addition, these transgenic plants showed enhanced resistance against the virulent fungus *Botrytis cinerea* (Seo et al. 2001). It has also been reported that the expression of the *pdf1.2* gene in transgenic *Arabidopsis* plants (Seo et al. 2001; Kumar et al. 2009; Zarei et al. 2011)

and avocado (Raharjo et al. 2008) may confer resistance to phytopathogens, which suggests a role of this defence protein in the defence system of these tissues.

Most of the reports on the expression of the *pdf1.2* gene as a defence response induced by MJ or its analogues have been studied in the vegetative tissues of *Arabidopsis*. This is the first study that reports the induction of this gene in papaya fruit in response to the exogenous application of MJ, which indicates the presence of similar defence mechanisms in both the vegetative and fruit tissues of plants.

This is the first study in which the induction of *pdf1.1* and *pdf1.2* gene expression is reported in papaya fruits 'Maradol' in response to the exogenous application of MJ. The *pdf1.1* and *pdf1.2* genes are part of the molecular defence mechanism that is activated by MJ, which explains the observed reduction in the incidence of fungal infections during the postharvest storage of MJ-treated fruits. The results of this study allow us to propose that the molecular defence mechanisms in fruits are similar to those found in the vegetative tissues of plants.

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