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Research article

Genotypic and environmental effects on the level of ascorbic acid, phenolic compounds and related gene expression during pineapple fruit development and ripening

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ABSTRACT

Pineapple (*Ananas comosus* (L.) Merr.) is a non-climacteric tropical fruit whose ripening could be accompanied by oxidative processes and the concurrent activation of enzymatic and non-enzymatic reactive oxygen species (ROS) scavenging systems. To better understand the variability of these processes among climatic environments or genotypes in pineapple, the temporal expression dynamics for genes encoding oxidative and antioxidative stress enzymes were analyzed by real-time RT-PCR during fruit development and ripening, among three cultivars: Queen Victoria, Flhoran 41 and MD-2 hybrid, and in two climatic areas. Pineapple development and ripening involved changes in the levels of transcripts encoding for polyphenol oxidase and transcripts involved in the first steps of the phenylpropanoid pathway and in the balance of ROS, especially those encoding for ascorbate peroxydase and metallothioneins, regardless of the cultivar. Our results confirm the same dynamic in gene expression from the two environmental crop areas, however climatic conditions influenced the level of the expression of the major transcripts studied that were linked to these oxidative and antioxidant metabolisms. MT3a and MT3b transcripts were not influenced by genetic factor. The genetic effect was not significant on the various transcripts linked to the first steps of the phenylpropanoid pathway and to phenol oxidation, except 4CL ones. In ripe pineapple, highly significant relationships were found between the contents in antioxidant metabolites, i.e., ascorbic acid and total phenolic compounds, and the transcript levels of genes involved in the enzymatic ROS-scavenging system and in the biosynthesis or regeneration of ROS-scavenging compounds, like phenylpropanoids, ascorbic acid, metallothioneins.

1. Introduction

Pineapple *Ananas comosus* (L.) Merr. is a multiple fruit consisting of coalesced berries. It is currently the third most important tropical fruit after banana and mango in terms of worldwide production. Pineapple is cultivated mainly for fresh or canned fruit and juice. Pineapple flesh is rich in biologically-active substances such as vitamins and phenolic compounds (Gil et al., 2006; Montero-Calderón et al., 2010), which are important for the human diet due to their antioxidant and anti-inflammatory properties (Poiroux-Gonord et al., 2010). Physicochemical characteristics involved in pineapple quality, including antioxidants, evolve during the 3–5 months of fruit development. The optimal quality is reached at a full maturity stage that corresponds to the extent of the yellow or red-orange skin color area, depending on the cultivar, and to a high sweetness-to-acidity balance and aroma sensation (Brat et al., 2004; Wei et al., 2011).

Fleshy fruits are generally classified into two physiological groups, climacteric and non-climacteric, according to their respiratory activity and associated ethylene biosynthesis profiles during ripening. Pineapple is an example of a non-climacteric fruit and can present oxidative processes during its ripening (Moyle et al., 2005), as observed for other non-climacteric fruits such as strawberry (Aharoni et al.,

Abbreviations: βtub, beta tubulin; 60sRP, 60s RP; MT3a, MT3 Metallothionein; MT3b, MT3 Metallothionein; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; PPO1, Polyphenol oxidase; PPO2, Polyphenol oxidase; CAT, Catalase; Fe-SOD, Fe Superoxide dismutase; [Cu/Zn]-SOD, [Cu/Zn] Superoxide dismutase; APx1, Ascorbate peroxidase; APx3, Ascorbate peroxidase; GR, Glutathione Reductase; MDHAR, Monodehydroascorbate reductase; QV, Queen Victoria; RL41, Flhoran 41; MD2, MD-2 hybrid

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2002) and grape (Pilati et al., 2007). In contrast to the well-studied climacteric fruits such as tomato, apple and banana, the process of development and ripening of non-climacteric fruits is less well known (Cherian et al., 2014). This oxidative processes require an increase in reactive oxygen species (ROS), which are highly reactive molecules that may cause oxidative damage to biological macromolecules, including proteins, DNA and lipids (Apel and Hirt, 2004). Various cellular enzymatic and non-enzymatic mechanisms have the capacity to scavenge ROS to modulate their steady-state concentrations, avoiding their potential toxicity while allowing them to function as signal molecules (Mittler et al., 2004). A few studies have reported that the ripening of non-climacteric fruit is accompanied by, in particular, the accumulation of transcripts of genes involved in the enzyme-mediated scavenging system and in the antioxidant compound metabolism and regeneration (Aharoni and O'Connell, 2002; Pilati et al., 2007). Further studies are required to analyze genotypic and environmental effects on expression profiling of these ripening-related genes, as described in this current report.

The enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), catalase (CAT), the ascorbate peroxidase, and the ascorbate-glutathione cycle (Apel and Hirt, 2004). Indeed, hydrogen peroxide can be converted into water, involving the oxidation and the regeneration of ascorbate, glutathione and NADPH by enzymes such as ascorbate peroxydase (APX), gluthatione reductase (GR) and monodehydroascorbate reductase (MDHAR), as described by Apel and Hirt (2004). The levels of the transcripts of genes involved in the enzymatic detoxification of ROS have been shown to be modulated during non-climacteric ripening, especially those of APX and SOD in grape and pineapple (Koia et al., 2012; Pilati et al., 2007), and those of CAT in citrus (Peroni et al., 2007), which were up-regulated.

In addition to enzymatic scavenging pathways, fruit cells have a network of low-molecular-mass antioxidant molecules such as ascorbate or phenolic compounds, which are also involved in non-enzymatic protection against oxidative stress and damage caused by ROS. Ascorbic acid is one of the most frequently studied and powerful antioxidants (Smirnoff, 2000). Phenolic compounds possess ideal structural chemistry for free radical and hydrogen peroxide scavenging activities, and for altering peroxidation kinetics (Takahama and Oniki, 1997). These antioxidants can work together to limit oxidative stress (Takahama and Oniki, 1997). H_2O_2 can also be reduced by peroxidases using phenolics as primary electron donors, making it possible for ascorbic acid to reduce phenoxyl radicals generated by this oxidation. The levels of these antioxidants have been reported to vary during fruit ripening. An increased contribution of the reduced form of ascorbate to the total ascorbate pool was observed in berries at the latter stages of ripening (Melino et al., 2009). At these stages, the expression of MDHAR and dehydroascorbate reductase (DHAR) genes encoding ascorbic acid recycling enzymes was up-regulated. Studies on grape berries and strawberries, two non-climacteric fruit models that accumulate phenolic compounds, reported that fruit ripening was associated with the synthesis of metabolites from the phenylpropanoid pathway, assisted by the up-regulation of phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) genes, involved in the first three committed steps of the pathway (Almeida et al., 2007; Sweetman et al., 2012).

The phenolic composition of fruits may be modified by oxidative reactions involved in the antioxidant activity of the phenols and oxidative browning. In fact, phenolic compounds are substrates for enzymatic browning reactions that are initially induced by enzymatic oxidation of phenolic compounds by polyphenol oxidases (PPO) and that form colored quinones. These reactions significantly diminish consumer acceptance, storage life and the value of the fruit products, especially in the case of pineapple, which is known to develop browning symptoms (Raimbault et al., 2010; Zhou et al., 2003a). However, high levels of PPO activity reduce the free-oxygen level available for ROS production and may be one of elements that contribute to the lowering of the cellular ROS level during stress conditions (Ortega-García and Peragón, 2009). Thus, the induction of PPO expression has been linked to fruit tolerance to stress conditions. Indeed, the two PPO genes identified in pineapple have been shown to be highly up-regulated in response to chilling and wounding (Stewart et al., 2001).

Another group of low-molecular-weight compounds are metallothioneins, small cysteine-rich proteins that provide thiols for metal chelation. Metallothioneins are not only involved in maintaining homeostasis of essential metals and metal detoxification, but are also involved in a range of physiological processes, including ROS scavenging (Mir et al., 2004). Various studies have shown that the expression of metallothionein genes increased during fruit development and was highest in mature fruit (Moyle et al., 2005; Pandit et al., 2010). These authors suggested that metallothioneins may be involved in scavenging ROS generated during fruit ripening.

The molecular mechanisms involved in non-climacteric fruit ripening have been studied in grape (Pilati et al., 2007), pea (Matamoros et al., 2010) and strawberry (Aharoni et al., 2002) to gain a broader knowledge about the variability of fruit quality and maturity. Recent studies on pineapple analyzed changes in gene expression during ripening of Smooth Cayenne cv (Koia et al., 2012) and involved in the cold response (Chen et al., 2016). Little is known about the effects of the climatic environments and of the genotypes on the expression of genes related to the oxidative balance since non-climacteric fruits may contain a transcriptional program responsive to oxidative stress induced during ripening (Aharoni and O'Connell, 2002). A better understanding would contribute to adjusting growing practices according to the cultivar and the climatic area in order to produce high-quality fruit in a changing environment. The temporal dynamics of the expression of genes coding oxidative and antioxidative stress enzymes were therefore analyzed in pineapple fruit. For this purpose, the levels of transcripts of several ROS scavenging enzymes and of antioxidant molecule synthesis and recycling were identified during the development and ripening of pineapple. To evaluate the seasonal and cultivar-specific variations, three cultivars, Queen Victoria, Flhoran 41 and MD-2 hybrid, for which no molecular markers approach exists, and two climatic areas were studied at different development stages. In ripe fruits, the relationships between antioxidant compound content, i.e., ascorbic acid and total phenolic compounds, and transcript levels related to oxidative stress and antioxidant system are discussed.

2. Materials and methods

2.1. Experimental sites and fruit material

Pineapple fruits (*Ananas Comosus* Merr L.) from three cultivars, Queen Victoria (QV) clone 'RE43', Flhoran 41 (RL41) clone 'RL41', and MD-2 hybrid (MD2) clone 'DLNE', were grown in two different climatic areas in Reunion Island. The first field was located in the southwest of the island at CIRAD's Bassin Plat Research Station, St Pierre, 150 m above sea level (55°29′20.64″E, 21°19′21.62″S), corresponding to a drier climatic area than the eastern one, with a cumulative rainfall of 380 mm and an average temperature of 24.1 °C during the period of fruit growth studied. The second field was located at 290 m above sea level in the east of the island, St Benoit (55°42′12.86″E, 21°05′53.85″S), corresponding to a very wet area with a cumulative rainfall of 1680 mm and an average temperature of 21.7 °C during the period of fruit growth studied. Each field was identically managed following the locally recommended cultural practices (Fournier, 2011).

Fruits from QV of the East field were not studied due to the risk of the development of fruitlet core rot disease in this climatic area, due to *Fusarium ananatum* that is known to involve a response in the accumulation of phenolic acids (Barral et al., 2017).

Fruits were harvested at five development stages from 30 to 140 days after flowering. Flowering was defined as 50% of inflorescences with at least one open corolla. The different developmental stages were