Expression patterns of ethylene biosynthesis genes from bananas during fruit ripening and in relationship with finger drop

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Abstract

Background and aims

Banana finger drop is defined as dislodgement of individual fruits from the hand at the pedicel rupture area. For some banana varieties, this is a major feature of the ripening process, in addition to ethylene production and sugar metabolism. The few studies devoted to assessing the physiological and molecular basis of this process revealed (i) the similarity between this process and softening, (ii) the early onset of related molecular events, between the first and fourth day after ripening induction, and (iii) the putative involvement of ethylene as a regulatory factor. This study was conducted with the aim of identifying, through a candidate gene approach, a quality-related marker that could be used as a tool in breeding programmes. Here we examined the relationship between ripening ethylene biosynthesis (EB) and finger drop in order to gain further insight into the upstream regulatory steps of the banana finger drop process and to identify putative related candidate genes.

Methods

Postharvest ripening of green banana fruit was induced by acetylene treatment and fruit taken at 1–4 days after ripening induction, and total RNA extracted from the median area [control zone (CZ)] and the pedicel rupture area [drop zone (DZ)] of peel tissue. Then the expression patterns of EB genes (\textit{MaACO1}, \textit{MaACO2}, \textit{MaACS1}, \textit{MaACS2}, \textit{MaACS3} and \textit{MaACS4}) were comparatively examined in CZ and DZ via real-time quantitative polymerase chain reaction.

Principal results

Differential expression of EB gene was observed in CZ and DZ during the postharvest period examined in this study. \textit{MaACO1}, \textit{MaACS2} and \textit{MaACS1} were more highly induced in DZ than in the control, while a slight induction of the \textit{MaACS4} gene was observed. No marked differences between the two zones were observed for the \textit{MaACO2} gene.

Conclusions

The finger drop process enhanced EB gene expression including developmental- and ripening-induced genes (\textit{MaACO1}), specific ripening-induced genes (\textit{MaACS1}) and wound-induced

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genes (MaACS2). Thus, this process might be associated with a specific ethylene production in DZ of the pedicel area and the result of crosstalk between developmental, ripening and wound regulatory pathways. MaACO1, MaACS1, MaACS2, and to a lesser extent MaACS4 genes, which are more highly induced in DZ than in CZ, could be considered as putative candidates of the finger drop process.

Introduction

Ethylene is a gaseous plant hormone that regulates many developmental events and biotic and abiotic stress responses of plants. Banana fruits undergo a climacteric ripening process. This is characterized by a ripening-related increase in respiration and a burst of ethylene production concomitantly with physicochemical and biochemical changes, including chlorophyll breakdown, increased starch degradation and sugar synthesis, and fruit softening.

Finger drop is a key feature that is closely associated with ripening of some banana varieties. This phenomenon has a substantial economic impact for the banana marketing sector. Indeed, bananas are marketed in hands of generally 4–9 fruits. Dislodgement of individual fruits from the hand at the pedicel area considerably reduces the commercial value of the product because hands with missing fingers or fingers without pedicels cannot be sold to consumers. Despite this economic importance, very few studies have been devoted to this phenomenon.

The finger drop process was first observed in bananas of the Cavendish subgroup in 1934 (Hicks 1934). It was defined as physiological softening and weakening, thus causing individual fruit in a hand to separate from the crown (Baldry et al. 1981). The sensitivity to finger drop within Musa germplasm varies according to the variety and ploidy (New and Marriott 1983; Pereira et al. 2004), growing and postharvest ripening, and storage conditions (Semple and Thompson 1988; Paull 1996; Saengpook et al. 2007). At the biochemical level, changes in water-soluble pectin, i.e. a cell wall polysaccharide component, have also been reported to be associated with finger drop. In addition to the activities of some cell wall hydrolases including pectate lyase and polygalacturonase, an increase in water-soluble pectin has been observed in the drop zone (DZ) as compared with control fruit (Imsabai et al. 2006). Recent molecular studies performed in Cavendish bananas also showed that a change in the expression of major cell-wall-modifying genes occurs specifically in the finger drop area (Mbéguié-A-Mbéguié et al. 2009). Overall, major molecular changes in the expression of genes coding for cell-wall-modifying proteins (CWMPs) occurred 1–4 days after ripening induction, but in a sequential manner. Firstly, there were changes in the expression of pectolytic and cell-wall-loosening genes, mainly during Days 1–2, followed by changes in the expression of xyloglucan genes, mainly during Days 3–4. The fact that some CWMP genes are involved in both finger drop and the fruit-softening process, with transcriptional regulation by ethylene, suggests that these two processes that occur during banana fruit ripening might involve common regulatory mechanisms and factors, with ethylene being one of them.

Numerous physicochemical, biochemical and molecular findings have shown that fruit softening is one of the ripening physiological processes that is most sensitive to ethylene (Gerasopoulos and Richardson 1996; Jiang et al. 1999; Kim et al. 1999; Flores et al. 2001; Grimplet 2004; Hayama et al. 2006; Johnston et al. 2009).

In higher plants, including banana, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) catalyse the two key steps of the ethylene biosynthesis (EB) pathway: the formation of methionine via S-adenosyl-l-methionine (AdoMet) and the cyclic non-protein amino acid ACC, respectively. In banana, ethylene production displays a sharp peak at the onset of ripening, followed by a rapid decrease thereafter due to a slight decline in the ACC content and a sharp decrease of in vivo ACO activity attributed to the availability of its cofactors, especially iron and ascorbate (Liu et al. 1999; Choudhury et al. 2008).

The ACS and ACO proteins have been widely studied at both biochemical and molecular levels in different species. ACC oxidase and ACS are encoded by a multi-locus family whose members are differentially regulated at transcriptional and translational levels by various developmental environmental and hormonal signals (Bleecker and Kende 2000; Wang et al. 2002; Lin et al. 2009). At least nine ACS and three ACO genes have been isolated from banana and published in the literature or directly registered in the GenBank database (Liu et al. 1999; Huang et al. 2006; Inaba et al. 2007). However, only a few of them were fully characterized in regard to ripening and none in regard to the finger drop process. The ACS and ACO genes are transcriptionally and differentially regulated according to the tissue
and stimuli. One ACS (MaACS1) and two ACO (MaACO1 and MaACO2) genes were found to be ripening related (Liu et al. 1999; Huang et al. 2006; Inaba et al. 2007). Post-translational regulation has also been reported in several plants, and mainly for ACS enzymes. This regulation is based on the C-terminal region of the ACS protein that can be directly phosphorylated by protein kinase, leading to an increase in protein stability (Lin et al. 2009; Han et al. 2010; Kamiyoshihara et al. 2010; Skottke et al. 2011; Wang et al. 2011; Choudhury et al. 2012), or bound to a substrate-specific adaptor, i.e. the ETO1 protein, and directed to the ubiquitin (Ub)/26S proteasome system (Chae and Kieber 2005; Yoshida et al. 2005). The ACS activity enzyme encoded by a ripening-related gene MaACS1 from banana was also subjected to post-translational regulation during fruit ripening. A few studies also reported a proteolytic cleavage of the protein leading to a decrease in the apparent pI of the protein and its inactivation, suggesting that this protein might also be under post-translational regulation as was ACS protein (Barlow et al. 1997; Ramassamy et al. 1997).

In this study, we investigated, at the transcriptional level, the putative relationship between the finger drop process and EB, i.e. two processes that occur during banana fruit ripening. To this end, the transcript abundance of ACS and ACO genes from banana was estimated comparatively in the median zone and DZ during the ripening of bananas harvested at the commercial maturity stage. Our findings suggest a possible role of ethylene and ripening-regulated elements in the regulation of the finger drop mechanism.

**Methods**

**Fruit harvesting and ripening induction**

The banana fruits (Musa acuminata, AAA, Cavendish, cv Grande Naine) used in this study were collected from at least four banana plants taken randomly from a banana farm near the CIRAD research station (elevation 250 m; andosol; rainfall 3500 mm/year), Guadeloupe (French West Indies). Banana plants were grown under conventional field practices and, on the basis of heat concept unit (Umber et al. 2011), fruits were harvested at commercial maturity stage [i.e. 900 degree-day (dd)], which corresponds to ~90 days after flowering.

After harvest, internal fruit of the median hand of all banana bunches, considered to be comparable (Liu 1976), were pooled and kept for 24 h at 20 °C in chambers. Fruits were then placed into sealed Plexiglas boxes, and their ripening was induced by injection of 1000 p.p.m. acetylene and the boxes were kept for 24 h at 20 °C and ambient humidity. At the end of treatment, fruits were removed from the boxes and kept ripening at 20 °C in air and ambient temperature. During postharvest ripening, a sample of three fruits was taken daily to assess the postharvest ripening process and finger drop development.

**Assessment of the physiological stage of fruit and measurement of finger drop development during postharvest ripening**

The physiological stage of fruit was assessed through measurement of soluble solid content (SSC). To this end, 5 g of fresh powder of pulp tissue were homogenized in an equivalent volume of distilled water and the mixture was centrifuged for 10 min at 10 000 × g and 4 °C. The fruit juice was collected and the SSC was determined using a digital Refracto 30PX/GS refractometer from Mettler Toledo (Grosseron, Saint-Herblain, France) and expressed in Brix. The development of finger drop was measured as previously described (Chillet et al. 2008).

All experiments were performed on three fruits at each time point. Immediately after these analyses, peel tissue corresponding to the median part of the fruit [control zone (CZ)] and to the rupture area of the fruit pedicel (DZ), and pulp tissue were sampled separately, frozen in liquid nitrogen and stored at −80 °C until use for total RNA extraction and gene expression analysis.

**RNA extraction and quantitative real-time polymerase chain reaction analysis**

Total RNAs were extracted twice from a pooled sample tissue using a modified hot borate method (Wan and Wilkins 1994; Mbéguie-A-Mbéguie et al. 2008b). At each developmental stage, peel tissue from three fruits was pooled due to the small quantity of peel material obtained per fruit, mainly from the DZ, thus making it difficult to blend in a coffee grinder.

The relative expression of each transcript was determined in triplicate on two independent RNA extracts by quantitative real-time polymerase chain reaction (qPCR) using a 7500 Real-Time PCR System (Applied Biosystems, Courtaboeuf, France). The first-strand cDNA synthesis was performed from 2 μg of RQ1-DNase-treated RNA from each RNA extract using a random hexamer primer and MMLV reverse transcriptase (Promega, Charbonnieres, France) according to the manufacturer’s instructions. The synthesized cDNA was diluted 1:10 with distilled water, and 5 μL of the diluted cDNA and gene-specific primer were used as the template for qPCR analysis in a 20 μL volume reaction, as previously described in Mbéguie-A-Mbéguie et al. (2008a). All primer sequences used in this study are listed in Table 1.
The relative fold differences in expression of each gene between samples were determined using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001) with the actin gene as reference and the fruit CZ of peel tissue sampled at harvest before ripening induction used as calibrator.

**Results**

**Finger drop pattern during postharvest fruit ripening**

During postharvest ripening of acetylene-treated banana fruit, the SSC content estimated via the Brix value started to increase at Day 1 after ripening induction and increased progressively until Day 4, when it reached its maximum. The Brix value remained constant from Day 4 to Day 6 (Fig. 1). According to the rupture force measurement, Cavendish banana finger drop started 1 day after ripening induction and continued progressively throughout the postharvest ripening stage. However, a marked decrease was observed between Days 1 and 3 after ripening induction, with a more than 2-fold decrease in the pedicel rupture force. As the pedicel rupture force pattern is considered to be an effective way of measuring banana finger drop (Saengpook et al. 2007), our data suggest that our experimental conditions induced the development of the finger drop process in Cavendish bananas.

**Expression of EB genes in peel tissue from CZ and DZ during banana fruit ripening**

The expression profiles of six EB genes, including two ACO and four ACS, were studied during postharvest ripening of banana fruit harvested at the commercial mature green stage and treated with acetylene (Fig. 2).

No change was observed in the MaACO2 mRNA level in both CZ and DZ tissues. At harvest, the MaACS3 level was 2-fold higher in the DZ compared with the control. This level decreased markedly at Day 1 after ripening induction to reach a level comparable to that observed in the CZ, and then the MaACS3 mRNA level remained constant in both tissues. The other EB genes, i.e. MaACO1, MaACS1, MaACS2 and MaACS4, were highly and transiently induced in DZ compared with the control, MaACO1 and MaACS4 being the most and least expressed ones, respectively. For the MaACS1, MaACS2 and MaACS4 genes, mRNA accumulation peaked 2 days after ripening induction. MaACS4 was the unique gene presenting, at harvest time, a low transcript level in the DZ compared with the control.

### Table 1  Sequences of gene-specific primers used in this study

| Gene   | Primer name | Sequences                        | Annealing temperature | Product size (bp) |
|--------|-------------|----------------------------------|-----------------------|-------------------|
| MaACT  | Act-F       | GAGAAGATACAGTTGCTGGGA            | 60                    | 231               |
|        | Act-R       | ATTACATCGAAATATTAAAAAG           |                       |                   |
| MaACO1 | ACO1-F      | AAGCTCTAGTCGGCGGCATAA            | 60                    | 152               |
|        | ACO1-R      | GACAGCTTCCATACCGGAA             |                       |                   |
| MaACO2 | ACO2-F      | CCAAGGAACCCAGATTGGA             | 60                    | 125               |
|        | ACO2-R      | TGGTAGCTCCACAGATAGCA            |                       |                   |
| MaACS1 | ACS1-F      | AGAACTCTCTCTACTCTCGAT           | 60                    | 215               |
|        | ACS1-R      | ATGATAGTCCTGCAAAGTGG            |                       |                   |
| MaACS2 | ACS2-F1     | TGGGCCTTTGGTCTCCTGGG           | 60                    | 151               |
|        | ACS2-R1     | AACACACCCCGTCCCCTGCC           |                       |                   |
| MaACS3 | ACS3-F1     | CCGTACTATCCAGGTCGACAGGG         | 60                    | 231               |
|        | ACS3-R1     | GAAGTCGACGGGTTTGCCAGTCT        |                       |                   |
| MaACS4 | ACS4-F1     | GCAGGAGCGGTCGGCCCTAGGG         | 60                    | 166               |
|        | ACS4-R1     | CGAGTCAAGCTGGTGCCCG           |                       |                   |

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Discussion

Finger drop is a major fruit ripening feature for some banana varieties. Our findings confirmed this by showing a progressive decrease in rupture pedicel force, concomitantly with finger drop sensitivity, throughout fruit ripening, along with a decrease in the SSC, as expressed by the Brix value (Fig. 1). Our previous findings also showed that molecular mechanisms related to the banana finger drop phenomenon occurred earlier after ripening induction as was ripening ethylene production (Liu et al. 1999; Inaba et al. 2007), while no marked changes were observed in the expression of MaACS3, MaACS4 and MaACO2 genes in CZ, suggesting that these genes might be less important during ripening of banana peel tissue. In contrast with previous studies showing that MaACS2 gene was expressed only in banana pulp tissue and upon wounding (Liu et al. 1999), our data showed a low but transient induction of MaACS2 in control zone of peel suggesting that this gene was ripening regulated in peel tissue. The discrepancy between the two data may be due to the analytical method, i.e. northern blot used by Liu et al. (1999) and qPCR used in this study.

The finger drop process enhanced EB gene expression, including the MaACO1, MaACS1, MaACS2 and MaACS4 genes, of which the corresponding mRNA was accumulated to a great extent in DZ compared to the control. Therefore, we hypothesized that the finger drop process is associated with ethylene production occurring specifically in the DZ with a putative involvement of one ACC oxidase (MaACO1) and three ACC synthase (MaACS1, MaACS2 and MaACS4) genes. However, this hypothesis needs to be validated through the measurement of ethylene produced by peel tissue taken from DZ in comparison to that taken from the median zone. The MaACO1 and MaACS2 genes previously identified as wound-inducible genes in pulp and leaf banana tissues (Liu et al. 1999; Mbéguie-A-Mbéguie et al. 2008a) were also transcriptionally enhanced by finger drop. Although the tissues and physiological stages examined in these previous studies are different, our data suggested that finger drop might also imply a wound-related mechanism. Considering that ripening and wounding processes are both associated with ethylene production, it should be interesting to assess whether finger drop, wounding and ripening share some ethylene transduction pathway components.

ACS and ACO genes are encoded by a large and small multigenic family, respectively. In contrast to ACO, the members of which are highly conserved, the ACS multigenic family was classified into three types according to the consensus motifs present at their C-terminal poly-peptide (Yoshida et al. 2005). It has been stated that individual members of the ACS and ACO multigenic families were not restricted to only one function. In order to assess the relationships between the structure
of ACS and ACO polypeptides and their putative function, an unrooted phylogenetic tree was constructed from a multiple alignment of ACS and ACO polypeptides registered in the database (Fig. 3). Three major lineages can be discerned from the ACS phylogenetic tree (Fig. 3A). MaACS1 belongs to Type 1 ACC synthase. Indeed, the MaACS1 C-terminal region presents the main features of Type 1 ACS protein including the Ser residues in the ‘RLSF’ motif, necessary for CDPK phosphorylation (Chae and Kieber 2005), followed by a 27-amino-acid tail containing the two Ser residues 476 and 479 recently proved to be phosphorylated during banana fruit ripening (Choudhury et al. 2010, 2012), and finally the absence of the ‘WVF’ binding ETO1 motif (Yoshida et al. 2005), which is degenerated to ‘WDEAL’. MaACS2, MaACS3 and MaACS4 polypeptide sequences are grouped in the same cluster, which is clearly divergent from MaACS1.

A previous study suggested that MaACS2, MaACS3 and MaACS4 are members of the same subgroup of the ACS family (Liu et al. 1999; Inaba et al. 2007). Consistent with this, the phylogenetic tree includes MaACS2, MaACS3 and MaACS4 into a divergent subgroup family of Type 2 ACS. However, this needs to be confirmed with a phylogenetic tree constructed with the complete MaACS2, MaACS3 and MaACS4 polypeptide sequences, as those used here are partial and lack the last 80 amino acid residues. Two main subfamilies are observed for the ACO phylogenetic tree (Fig. 3B). Banana ACO genes examined in this study are grouped with the other major ACO genes, consistent with the high conservation of these proteins. Based on the present data (i.e. the limited number of ACO and ACS genes examined), a putative relationship between ACO and ACS lineages and their corresponding function in regard to finger drop cannot...
Fig. 3 Phylogenetic analysis of ACS (A) and ACO (B) sequences from banana and other plant species. The phylogenetic tree was constructed with the Gonnet residue weights and after a complete sequence alignment performed using the ClustalX algorithm (Jeanmougin et al. 1998). For multiple alignments, the gap opening penalty was 10, with a gap extension penalty of 0.2 and the delay of divergent sequences was set at 30%. The consensus tree was displayed using the TREEVIEW program (Page 1996). All banana sequences are indicated by underlining while those whose expression was examined in this study are in bold. ACC synthase sequences used for phylogenetic tree construction are:

- *Musa acuminata* [MaACS1 (AB021906), MaACS2 (AB021907), MACS2 (AF056162), MaACS3 (AB021908), MaACS4 (AB266314), MaACS5 (AJ223186)], *Arabidopsis thaliana* [AtACS2 (Q06402), AtACS4 (NP_179866), AtACS5 (AAAS0098), AtACS6 (Q50AR0), AtACS7 (AA48754), AtACS8 (AA50090), AtACS9 (AA48755)], *Cucumis sativum* [CsACS1 (BAA33374), CsACS2 (BAA33375), CsACS3 (BAA33376)], *Doritaenopsis* sp. [DsACS1a (L07882), DsACS1b (L07883)], *Lupinus albus* [LuACS1 (AF119411), LuACS2 (AF119412), LuACS3 (AF119413), LuACS4 (AF119410), LuACS5 (AF119414)], *Solanum lycopersicon* [SlACS1a (AAF08761), SlACS1b (AAF08761), SlACS2 (CA41855), SlACS3 (AA48945), SlACS4 (AAA0164), SlACS5 (BAA34923), SlACS6 (BAA34923)], *Solanum tuberosum* [StACS1a (Z27233), StACS1b (Z27234), StACS2 (Z27235)] and *Vigna radiata* [VrACS1 (CAA77688), VrACS6 (U34978)], *Vigna radiata* (U34978). ACC oxidase sequences used for phylogenetic tree construction are: *Musa acuminata* [MaACO1 (AY804252), MaACO2 (X95599), MaACO2 (AF030410)], *Arabidopsis thaliana* [AtACO1 (AF030410), AtACO2 (AF030410), AtACS3 (Q8H1S4), AtACS5 (Q43383), AtACS6 (AAAS0098), AtACS7 (AAAS0098), AtACS9 (AAAS0098), AtACS10 (Q9LSW6)], *Cucumis sativum* [CsACO1 (AB004875), CsACO2 (AB004875)], *Diospyros kaki* [DkACO1 (AB004875), DkACO2 (AB004875)], *Doritaenopsis* sp. [DsACO1 (Z27233), DsACO2 (Z27234)], *Solanum lycopersicon* [SlACO1 (X58273), SlACO4 (AB013101), SlACO5 (AJ715790)], *Solanum tuberosum* [StACO1 (AF384820), StACO2 (AF384821)] and *Vigna radiata* [VrACO1 (U06049), VrACO2 (AM180697)].
yet be established. A more detailed analysis of the expression of the members of both ACO and ACS family genes is necessary before assigning a functional homology to this lineage. This is now possible with the availability of the banana genome sequence (D’Hont et al. 2012).

Conclusions and forward look

In conclusion, our data showed that the finger drop process might be associated with ethylene production, implying a large number of EB genes. However, this hypothesis needs to be validated through the measurement of ethylene produced at DZ.

The finger drop process is probably a result of the crosstalk between ethylene (i.e. ripening and wounding) and developmental regulatory pathways. The ethylene transduction pathway model has been proposed and the related components identified (Cara and Giovannoni 2008). On the other hand, MADS-box genes have recently been identified as a major component in the molecular circuit of the developmental regulation of fruit (Vrebalov et al. 2002; Giovannoni 2004; Elitzur et al. 2010). It should be interesting to identify the ethylene and developmental transduction components involved in the finger drop process. This represents an interesting challenge to gain further insight into the banana ripening process and especially the physiological events occurring in banana peel tissue, whose ripening process clearly differs compared with that of pulp.

Finally, with the prospect of identifying ripening-related markers through a candidate gene approach,
MaACO1, MaACS1, MaACS2, and to a lesser extent MaACS4 genes that are induced in DZ to a greater extent than in CZ could be considered as putative candidates related to the upstream regulatory steps of the finger drop process. However, and before their use in molecular breeding schemes for banana improvement, these candidates need to be validated through functional studies using cultivars contrasting their tendency to develop finger drop.

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Contributions by the authors

D.M.-A-M. designed the project, performed all the molecular biology experiments, constructed the phylogenetic tree, analysed the qPCR data (Fig. 2) and wrote the manuscript. O.H. performed all the physicochemical experiments, including fruit sampling, treatment, monitoring of postharvest ripening and physicochemical data analysis described in Fig. 1.

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Conflict of interest statement

None declared.

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