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RESEARCH PAPER

Expression patterns of cell wall-modifying genes from banana during fruit ripening and in relationship with finger drop

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Abstract

Few molecular studies have been devoted to the finger drop process that occurs during banana fruit ripening. Recent studies revealed the involvement of changes in the properties of cell wall polysaccharides in the pedicel rupture area. In this study, the expression of cell-wall modifying genes was monitored in peel tissue during post-harvest ripening of Cavendish banana fruit, at median area (control zone) and compared with that in the pedicel rupture area (drop zone). To this end, three pectin methylesterase (PME) and seven xyloglucan endotransglycosylase/hydrolase (XTH) genes were isolated. The accumulation of their mRNAs and those of polygalaturonase, expansin, and pectate lyase genes already isolated from banana were examined. During post-harvest ripening, transcripts of all genes were detected in both zones, but accumulated differentially. *MaPME1*, *MaPG1*, and *MaXTH4* mRNA levels did not change in either zone. Levels of *MaPME3* and *MaPG3* mRNAs increased greatly only in the control zone and at the late ripening stages. For other genes, the main molecular changes occurred 1–4 d after ripening induction. *MaPME2*, *MaPEL1*, *MaPEL2*, *MaPG4*, *MaXTH6*, *MaXTH8*, *MaXTH9*, *MaEXP1*, *MaEXP4*, and *MaEXP5* accumulated highly in the drop zone, contrary to *MaXTH3* and *MaXTH5*, and *MaEXP2* throughout ripening. For *MaPG2*, *MaXET1,* and *MaXET2* genes, high accumulation in the drop zone was transient. The transcriptional data obtained from all genes examined suggested that finger drop and peel softening involved similar mechanisms. These findings also led to the proposal of a sequence of molecular events leading to finger drop and to suggest some candidates.

Key words: Banana, cell-wall, expansin, finger drop, *Musa*, pectolytic genes, quality, ripening, xyloglucan endotranglucosylase/hydrolase.

Introduction

Banana is the second ranking fruit crop in the world, with an annual world production of ~70 million metric tons (FAOstat, 2007 faostat.fao.org/site/567/DesktopDefault.aspx?). Banana fruit ripening is characterized by a burst of ethylene production and respiration concomitantly with physicochemical and biochemical changes, including chlorophyll breakdown, increased starch degradation and sugar synthesis, and fruit softening. Finger drop is one of the

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main features closely associated with the banana ripening process. This process, which was reported for the first time in the triploid Cavendish AAA group, involves physiological softening and weakening, thus causing individual fruit in a hand to separate from the crown (Hicks, 1934; Baldry *et al.*, 1981; Semple and Thompson 1988). Bananas are marketed in hands of generally 4–9 fruits. Dislodgement of individual fruits from the hand considerably reduces the commercial value of the product because hands with fingers missing or fingers without pedicels cannot be sold to consumers. The finger drop process thus reduces the economic value of bananas. Despite this important economic impact, few studies have been devoted to gaining an insight into the physiological mechanisms related to banana finger drop. Susceptibility to finger drop varies widely according to cultivars and their ripening conditions, with triploid cultivars being less susceptible than tetraploids (New and Marriot, 1983). Among triploid cultivars, Valery is less susceptible than Gros Michel, and Hom Tong (*Musa acuminata*, AAA) shows massive drop while Namwa (*Musa* sp., ABB) does not (New and Marriot, 1983; Saengpook *et al.*, 2007). In addition to cultivars, the level of fruit development ripening conditions (including ethylene, humidity, and temperature) also affect the susceptibility to banana finger drop (Semple and Thompson, 1988; Paull, 1996; Imsabai *et al.*, 2006). Studies on the physiological mechanisms of banana finger drop have only recently been carried out and, to our knowledge, performed only at the biochemical level. In a study on two varieties that display massive (Hom Tong variety) and no finger drop (Namwa variety), respectively, Imsabai *et al.* (2006) showed that peel water content and thickness did not affect the finger drop process. By contrast, differential changes in the water- and CDTA-soluble fractions, and in the insoluble pectin fraction at the peel rupture area in the Hom Tong variety indicated high pectin breakdown and degradation activity in this variety. The authors also showed that this pectin breakdown was mainly the result of pectate lyase and polygalacturonase enzymes, as their corresponding activities increased concomitantly in the peel rupture area of the Hom Tong variety as compared with those of the Namwa variety. It is now assumed that cell wall modifications that occur during fruit ripening correspond to a complex process involving different mechanisms, including enzymatic and non-enzymatic degradation of cell wall components (Dumville and Fry, 2003; Bennett and Labavitch, 2008), cell wall synthesis (Mitcham *et al.*, 1989, 1991; Greve and Labavitch, 1991; Huysamer *et al.*, 1997; Rose and Bennett, 1999) and/or loosening of the cell wall network mediated by non-enzymatic proteins like expansin (Brummell, 2006). Enzymatic degradation of cell wall components is the result of the induction, at both molecular and biochemical levels, of pectolic and/or non-pectolitic cell wall-modifying proteins (CWMPs) according to the specific class of polysaccharides used as substrate. The pectolytic enzymes include endo- and exo-polygalacturonases (endo- EC 3.2.1.15; exo- EC 3.2.1.67), pectate lyases (PEL; EC 4.2.2.2), pectin methylesterases (PME; EC 3.1.1.11), pectin acetylesterases (PAE; EC 3.1.1.–), β-galactosidases (EC 3.2.1.23), and α-L-arabinofuranosidases (EC 3.2.1.55) (Vicente *et al.*, 2007; Goulao and Oliveira, 2008). These enzymes are able to cleave or modify the nature of the polysaccharide backbone or to remove neutral sugars from branched side-chains. Non-pectolytic enzymes are responsible for hemicellulose modifications and include endo-1,4-β-glucanases (EGase; EC 3.2.1.4), endo-1,4-β-xylanases (EC 3.2.1.8), β-xylanases (EC 3.2.1.37), and xyloglucan endo-transglycosylase/hydrolases (XTH; EC 2.4.1.207). Many of these proteins have been investigated, at both molecular and biochemical levels, in relationship with banana fruit softening. A co-ordinated increase in mRNA abundance, *de novo* synthesis or activity of a number of putative cell wall-modifying enzymes that may be responsible for or contribute significantly to polymer modifications has been observed (Dominguez-Puigjaner *et al.*, 1997; Pua *et al.*, 2001; Marin-Rodriguez *et al.*, 2003; Trivedi and Nath, 2004; Asif and Nath, 2005; Zhuang *et al.*, 2006, 2007; Sane *et al.*, 2007).

As none of these genes have been examined in relation to finger drop, an attempt was made to investigate, at the molecular level, the putative relationship between the expression of some cell wall-modifying genes (CWMGs) and the finger drop process in order to identify: (i) the putative physiological mechanism of the cell wall metabolism involved in the finger drop process, and (ii) relevant genes involved for purposes of identification of functional molecular markers.

Banana is a complex species and attempts to improve the fruit quality through breeding strategies is complex and difficult to set up. Access to functional molecular markers derived from relevant genes associated with fruit quality traits should therefore provide a valuable and helpful resource for the development of new crossing strategies and the selection process. Moreover, gaining further insight into the molecular mechanisms that govern the elaboration of fruit quality traits and their regulation is a key prerequisite for the development of other strategies to improve banana quality traits like post-harvest technology and/or biotechnology approaches.

In the present study, the accumulation of mRNA genes encoding a broad array of enzymes known to hydrolyse or modify the fine structure of cell wall polysaccharides was examined, with the view of correlating particular mechanisms and genes with previously observed changes in cell wall composition during finger drop.

Materials and methods

Plant material

All tissues used in this study were harvested from three banana plants (*Musa acuminata*, AAA, Cavendish, cv. Grande Naine) grown at the CIRAD research station (elevation: 250 m; andosol; rainfall: 3500 mm $year^{-1}$), Guadeloupe (French West Indies). During growth, bunches

on banana plants were covered with blue plastic bags to hamper insect infestations, and to streamline the development of whole fruits on the bunch. Green fruits were harvested at commercial ripeness, as determined on the basis of the heat unit concept (Ganry and Meyer, 1975; Jullien *et al.*, 2008). At each harvesting time, only internal fingers of the median hand on the bunch, considered as comparable (Liu, 1976), were taken into account for each bunch. After harvest, all fruits were kept for 24 h at 20 °C in chambers ventilated with humidified air before treatment with 10 000 ppm of azethyl (95% nitrogen/5% ethylene) for 24 h at 20 °C and ambient humidity. From 1–7 d after treatment (DAT), a sample of three fruits was taken daily for finger drop measurement. Immediately at the end of each measurement, peel tissues corresponding to the median part of the fruit (control zone) and to the rupture area of the fruit pedicel (drop zone) (Fig. 1) were sampled, separately frozen in liquid nitrogen, and stored at –80 °C until use.

Measurement of finger drop variations during post-harvest ripening of fruit

Finger drop was estimated by measuring the pedicel rupture force using a TA-XT2 penetrometer and an adapted probe as follows. Banana fruit were attached 6 cm above a table with a large clip. The probe was pressed down to a depth of 40 mm and at a constant speed of 5 mm s^{-1}, at the pedicel until it separated from the fruit (Chillet *et al.*, 2008). The required force was expressed in Newtons (N). All of these measurements were performed in triplicate on three separate fruits and the experiment was repeat three times.

Isolation of banana fruit genes coding for xyloglucan endotransglycosylase/hydrolase-like (XTH-like) and pectin methylesterase-like (PME-like)

Partial cDNAs encoding banana XTH-like genes were amplified using the 3′ RACE-PCR method (Frohman, 1990). Total RNA was extracted separately from peel tissue from control and drop zones of banana fruit taken at commercial ripeness, and at 1–4 DAT, using the modified hot-borate method (Wan and Wilkins, 1994; Mbéguié-A-Mbéguié *et al.*, 2008*a*). According to the manufacturer's instructions, 2 µg of each RNA extract was DNAse-treated using RQI DNase (Promega, Charbonières, France) before being separately reverse-transcribed into cDNA using Oligo-dT_{17} primers (Frohman, 1990; see Supplementary Table S1 at *JXB* online) and AMV reverse transcriptase enzyme (Promega). These cDNA were pooled and 1 µl of this mixture was subjected to 3′ RACE-PCR with Xth-F1 and dT_{17}-adapter primers in a 25 µl reaction containing 1× *Taq* buffer (Eurobio, Courtaboeuf, France), 150 nM of dNTP, 1.5 mM of $MgCl_2$, 200 nM of each primer, and 1 U of *Taq* polymerase. Xth-F1 is a degenerated forward primer designed from a conserved HDE(I/M)DFEF region among plant XTH-like proteins (Table 1). The PCR program involved one cycle of 1 min at 94 °C for template denaturation and *Taq* activation followed by 40 cycles of 30 s at 94 °C for template denaturation, 1 min at 40 °C for primer annealing, and 2 min at 72 °C for template elongation. The isolated cDNA fragments (approximately 1100 bp) were cloned into pGEM-T vector using the pGEM-T Easy Cloning kit (Promega) and sequenced by Cogenics (Grenoble, France). GenBank accession numbers are FJ264506 (*MaXTH3*), FJ264507 (*MaXTH4*), FJ264508 (*MaXTH5*), FJ264509 (*MaXTH6*), FJ264510 (*MaXTH7*), FJ264511 (*MaXTH8*), and FJ264512 (*MaXTH9*).

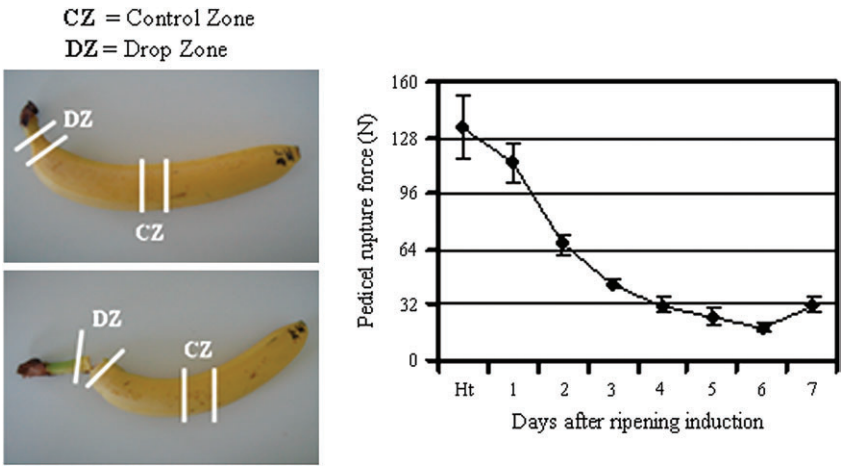

Fig. 1. Measurement of finger drop variations during post-harvest ripening of Cavendish banana through pedicel rupture force measurement. Banana fruits harvested at the mature green stage were stored overnight at 20 °C before treatment with 10 000 ppm of azethyl (5% ethylene+95% nitrogen) for 24 h. Fruits were further kept at 20 °C in air for 7 d to ripen. Banana finger drop was evaluated on the basis of the pedicel rupture force expressed in Newtons (N). This force was measured daily from the harvesting point (Ht) to day 7, as described by Chillet *et al.* (2008). Each data point is the mean of values obtained from at least four fruits originating from four replicate bunches. Vertical bars indicate standard deviation (SD). When no bar is shown, the SD is smaller than the symbol.

Table 1. Sequence analysis and comparison of banana PME genes

Analysis of nucleotide and polypeptide sequences of PME genes isolated in this study. The features of nucleotide and polypeptide sequences of *MaPME1* cDNA and those of the two genomic sequences of *MaPME2* and *MaPME3* are given in (A). A comparison of nucleotide and polypeptide sequences of *MaPME* genes (B), including determination of Scov and Qcov values, was performed using BLAST 2 (Tatusova and Madden, 1999).

(A)

Sequence	Gene or cDNA length (bp)	Polypeptide length (aa)	MW (kDa)	pI	Promoter length	Exon 1	Intron	Exon 2
MaPME1	1923	565	61	7.8	–	–	–	–
MaPME2	3365	559	61	6.7	1162	960	68	720
MaPME3	3186	574	62	7.06	1000	1029	81	696

(B)

Polypeptides	MaPME1: 1845 bp (565 aa)			
	Length	% identity (similarity)	Scov	Qcov
MaPME1	565	–	–	–
MaPME2	559	46 (60)	81.06	81.93
MaPME3	574	47 (59)	97.17	95.64

Nucleotides	CDS			
	Length	% identity	Scov	Qcov
MaPME1	1895	–	–	–
MaPME2	2135	75	9.3	8.2
MaPME3	2104	75	27.7	24.9

For pectin methylesterase genes, one partial cDNA and two genomic sequences coding for banana PME-like genes were isolated in this study. The partial cDNA sequence was isolated throughout EST sequencing. PME genomic sequences were identified throughout sequencing and annotation of BAC clones MAC077E20 (accession number AC226050) and MAC088K20 (accession number AC226051), isolated from a Cavendish BAC library (Piffanelli *et al.*, 2003).

Bioinformatic sequence analysis

The sequence similarity of the genes isolated in this study was determined using the online BLAST (Altschul *et al.*, 1997) and BLAST 2 sequence (Tatusova and Madden, 1999) programs on the National Center for Biotechnology Information BLAST website (National Library of Medicine, Bethesda, MD). For sequence comparison throughout the BLAST 2 program, two additional parameters were introduced, Qcov and Scov, which allows us to take into account the difference of the length between sequences. Query coverage (Qcov) is the ratio of length of the match by length of the query sequence. This value indicates the part of the query sequence covered by the alignment. Similarly, Subject coverage (Scov) was calculated as the ratio of length of the match by length of the subject or target length, and indicates the part of the subject or target sequence covered by the alignment.

All sequence alignments and phylogenetic tree construction were performed using the ClustalX algorithm with the Gonnet residue weights (Thompson *et al.*, 1997). For XTHs genes, the pair-wise alignment parameters used included a gap opening penalty of 35 and a gap extension penalty of 0.75. For multiple alignments, the gap opening penalty was 15, with a gap extension penalty of 0.3 and the delay divergent sequences was set at 25%. The consensus tree was drawn using the TREE VIEW program (Page, 1996). Search motifs in the promoter regions of *MA-PME* genomic sequences was found by screening the PLACE (Prestridge, 1991; Higo *et al.*, 1999) and PLANT CARE (Lescot *et al.*, 2002) databases. *In silico* prediction of 5′- and 3′-UTR regions of *MA-PME* genes and prediction of signal peptides were performed using EuGène 3.2 with rice parameters (Foissac *et al.*, 2008) and SignalP 3.0 (Bendtsen *et al.*, 2004) programs, respectively.

Oligonucleotide primers for gene expression through real-time qPCR and end-point PCR analysis

The oligonucleotide primers used for RT-qPCR analysis were designed using the Primer 3 freeware program (Roten and Skaletsky, 2000) according to the default criteria and the corresponding sequences are described in Supplementary Table S1 at *JXB* online. Except for polygalacturonase genes *MaPG3* and *MaPG4*, whose primers were designed

within the divergent coding region, all other primers were designed within the 3′-UTR region. The lengths of all PCR products ranged from 125 bp to 247 bp. The gene specificity of all primer sets was tested using the following three independent procedures: (i) BLAST searches against GenBank databases were performed for each primer to check that no member of a primer set matched another sequence in the banana genome; (ii) the specificity of PCR amplification was examined by monitoring the dissociation curves during qPCR reactions using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Courtaboeuf, France); (iii) individual PCR products were separated on 2% agarose gels and stained with ethidium bromide to examine their size and ensure that a single PCR product was detected for each primer pair, in accordance with the single peak observed in the dissociation curve. As for the *MaPME2* and *MaPME3* genes, the corresponding specific primers were designed based on the 3′-UTR predicted from the corresponding genomic sequence. The generated PCR fragment was further cloned and sequenced to confirm that it corresponded to a PME genomic sequence.

Real-time qPCR analysis

All qPCR experiments were performed on an ABI 7000 sequence detection apparatus (Applied Biosystems, Courtaboeuf, France) using the SYBR Green PCR Master Mix as described by Mbéguié-A-Mbéguié *et al.* (2008*b*). Two micrograms of total RNA extracted from different banana fruit tissues and organs treated with RQ1 DNase (Promega, Charbonnières, France) to remove possible contaminating genomic DNA were used. First-strand cDNA was synthesized from these RNA using AMV reverse transcriptase enzymes (Promega, Charbonnières, France) and random hexamers according to the manufacturer's instructions. To hamper the effect of putative PCR inhibitors that might be present in the reverse transcriptase reaction, as well as pipetting errors, the cDNA was 10-fold diluted with distilled water, and 5 µl of the diluted cDNA was used as a template for qPCR analysis. The qPCR program included one cycle of 5 min at 94 °C for template denaturation and *Taq* activation, followed by 40 cycles of 30 s at 94 °C for template denaturation, 15 s at 50 °C for primer annealing, and 30 s at 72 °C for primer elongation. Fluorescence was analysed using Sequence Detection Software (Applied Biosystems Courtaboeuf, France). A cycle threshold value (Ct) was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot.

The amplification efficiency of all primer sets was determined by qPCR analysis as described previously. To this end, we used 6 log of 2-fold serial dilutions made from a pool of cDNA reverse-transcribed from a mixture of RQI DNase-treated mRNA from different banana fruit tissues and organs. For each dilution, a qPCR was run in triplicate. The efficiency (slope) ranges were 83–100% (3.3–3.7), 87–100% (3.3–3.6), 73.3–77.3% (4.02–4.1), and 72–92% (3.12–4.2) for expansin, pectin methylesterase, polygalacturonase, pectate lyase, and xyloglucan endotransferase/hydrolase genes, respectively. They were measured using the Ct slope method (data not shown) and the formula $E_x=10^{(-1/\text{slope})}-1$.

The relative fold differences in expression of each gene between samples were determined using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). For each gene and sample, a qPCR was performed in triplicate on two independent RNA extracts as described above. The *MA-ACT* gene was used as the internal control to standardize the difference between template amounts and fruit tissues taken at harvest before ripening induction was used as the calibrator.

Results

Finger drop evolution of banana fruit measured via the pedicel rupture force

Banana fruits were harvested at commercial harvest grade corresponding to approximately 90 days after flowering (DAF). According to the rupture force measurement (Fig. 1), Cavendish banana finger drop started 1 d after ripening induction and continued progressively throughout the post-harvest ripening stage. However, a marked decreased was observed between days 1 to 3 after ripening induction, with more than a 2-fold decrease in the pedicel rupture force. As the pedicel rupture force pattern is considered to be a way of measuring banana finger drop (Saengpook *et al.*, 2007), our data suggest that our experimental conditions led to the development of the finger drop process of Cavendish banana.

Cloning of MaXTH *genes from banana fruit and sequence analysis*

Seven partial cDNA fragments encoding XTH-like polypeptides were isolated from of banana peel tissue. As two banana XTH homologues, *MaXET1* and *MaXET2*, were previously isolated and registered in the GenBank database (Lu *et al.*, 2004), the XTH cDNAs isolated in this study were named *MaXTH3* to *MaXTH9*. The sequence homology between *MaXTH* cDNA ranges from 3.1% to 71% at the nucleotide level and from 12% to 81% at the amino acid level, with *MaXTH5* and *MaXTH6* being highly homologous pairs. Compared with *MaXET1* and *MaXET2* genes, a high homology was observed with *MaXTH7* and *MaXTH4*, respectively. Analysis of the predicted *MaXTH* polypeptides revealed that they contained a DEIDFEFLG motif conserved within glycosyl hydrolase family 16 (GH16) enzymes and, adjacent to the putative active site, the potential N-linked glycosylation site N-X-S/T motif. For *MaXTH5* and *MaXTH6*, this last motif was substituted by the N-V-R motif which is characteristic of group-3 XTHs (Campbell and Braam, 1999; Johanson *et al.*, 2004; see Supplementary Fig. S1 at *JXB* online).

A phylogenetic tree was constructed to investigate the evolutionary relationships of XTHs polypeptides from the

partial banana cDNA and those from rice, tomato, and *Arabidopsis* (Fig. 2). Our data showed an organization of XTH proteins into two major clusters (I and II) as observed with rice XTH proteins (Yokoyama *et al.*, 2004), another monocotyledonous species as banana. A classification into four XTH families was observed in *Arabidopsis*, tomato, two dicotyledonous species, and in a broad range of plant species (Nishitani, 1997; Campbell and Braam, 1999; Rose *et al.*, 2002; Saladié *et al.*, 2006). Compared with this organization, our data indicated that cluster II mainly consisted of XTH proteins of family 2 of dicotyledons together with *MaXTH4* and *MaXTH9*. Cluster I can be divided into two subclusters. The first one included *MaXET1* and *MaXET2*, and *MaXTH3*, *MaXTH5*, *MaXTH6*, *MaXTH7*, and *MaXTH8*. It mainly consisted of XTH proteins of families 3 and 4 of dicotyledons. The second one mainly consisted of XTH proteins of families 1 and 2 of dicotyledons.

Cloning MaPME *genes from banana fruit and sequences analysis*

Three genes encoding a putative banana PME polypeptide have been isolated by *Musa* sequence data mining. *Musa* EST sequences produced one partial cDNA sequence (*MaPME1* accession number FJ264505). A similarity search in available *Musa* BAC sequences produced two BAC sequences: *MaC077E20* (GenBank accession number AC226050) and *MaC088K20* (GenBank accession number AC226051) of 177 729 bp and 152 711 bp, respectively. Automatic and manual annotation of these BACs showed that they contained 26 and 19 predicted genes in addition to 1 and 3 repetitive elements, respectively. The two BACs sequences are schematically shown in Supplementary Fig. S2 at *JXB* online. They are collinear and share an overlapping region of 130 kbp. Both sequences contained one common identical putative PME gene *(MaPME2)*. MaC077E20 contained an additional putative PME gene (*MaPME3)*. Only the putative complete *MaPME2* and *MaPME3* genes from BAC MaCO77E20 were subsequently used in this study. The features of *MaPME* cDNAs and genomic sequences are described in Table 1A. The putative promoter regions of both *MaPME2* and *MaPME*3 genes were analysed. They contained numerous *cis*-regulatory sequences that might be involved in tissue-specific or developmentally-regulated gene expression (see Supplementary Table S2 at *JXB* online). A sulphur-responsive element that contains the auxin-response factor (ARF) binding sequence (GAGACA) and the copper/O_2-reponsive element appeared to be specific to *MaPME2* and *MaPME3* promoter sequences, respectively.

The predicted coding region of *MaPME1* cDNA, *MaPME2* and *MaPME3* genomic sequences were compared at both nucleotide and polypeptide levels (Table 1B). *MaPME2* and *MaPME3* genes shared 7% identity at the nucleotide level, while at the amino acid level, they showed 38% and 56% identity and similarity, respectively. Compared to *MaPME1* cDNA, *MaPME2* and *MaPME3* genomic sequences diverged at the nucleotide level. Indeed, the high percentage of identity (75%) observed between these sequences is not significant as it was obtained in a short portion of their nucleotide sequence, as shown by the low Scov and Qcov values (less than 10% with *MaPME2* and 25% with *MaPME3*). At the polypeptide level, *MaPME2* and *MaPME3* shared 46% and 60% identity and similarity, respectively, with polypeptides predicted from *MaPME1* cDNA. The predicted polypeptides of the three *MaPME* genes presented features that are conserved in all PME polypeptides. Among these, there are the pre- and pro-sequences at the N-terminal extension, and five polypeptide segments that are characteristic of the C-terminal catalytic region, which constitutes the mature PME enzyme: GxYxE, QAVAL, QDTL, DFIFG, LGRPW (Micheli, 2001; Markovic and Janecek, 2004; see Supplementary Fig. S2 at *JXB* online). The pre-region or signal peptide required for protein targeting to the endoplasmic reticulum is cleaved in the first step of maturation. According to the SignalP 3.0 program (Bendtsen *et al.*, 2004); it was localized between amino acid residues 24–25 and 27–28 for *MaPME2* and *MaPME3*, respectively. Pro-sequence cleavage of the covalently attached mature enzyme may occur in the second step of maturation close to the RR(K)LL(M) motif (Markovic and Janecek, 2004; see Supplementary Fig. S2 at *JXB* online), thus leading to pro-sequence lengths of 200 and 217 amino acid residues for *MaPME2* and *MaPME3*, respectively. Based on the *Arabidopsis* PME sequence analysis, PME genes have been classified into two types: type I and II, having a long N-terminal pro-region and a short or absent pro-region, respectively. The role of the pro-region is currently unknown (Micheli, 2001; Markovic and Janecek, 2004; Giovane *et al.*, 2004; Pelloux, 2007). The long N-terminal pro-sequence of *MaPME2* and *MaPME3* predicted polypeptides suggests that both genes belong to type I.

Expression of pectolytic genes in peel tissues from control and drop zones during banana fruit ripening

Three classes of pectolytic genes were examined in peel tissue during banana fruit ripening and in relation to finger drop. Among these, three genes encode pectin methylesterase *MaPME1* to *MaPME3*, four encode polygalacturonase (*MaPG1 to MaPG4*; Asif and Nath, 2005), two encode pectate lyase (*MaPEL1* and *MaPEL2*; Dominguez-Puigjaner *et al.*, 1997; Pua *et al.*, 2001), and four encode expansin protein (*MaEXP1*, *2*, *4*, and *5;* Sane *et al.*, 2007). Gene expression experiments were performed in triplicate on two independent RNA extracts and gave the same results.

Pectolytic genes examined in his study were differentially expressed during ripening and in both the control and drop zones (Fig. 3). *MaPEL1* and *MaPEL2*, and *MaPG4* genes were the most expressed genes compared with the others. Among the three *MaPME* genes, *MaPME1* was the least expressed in peel tissue compared with the other two. Its expression decreased during ripening in both zones (Fig. 3A). By contrast, *MaPME2* was the most expressed in the drop zone. In the control zone, its mRNA level remained

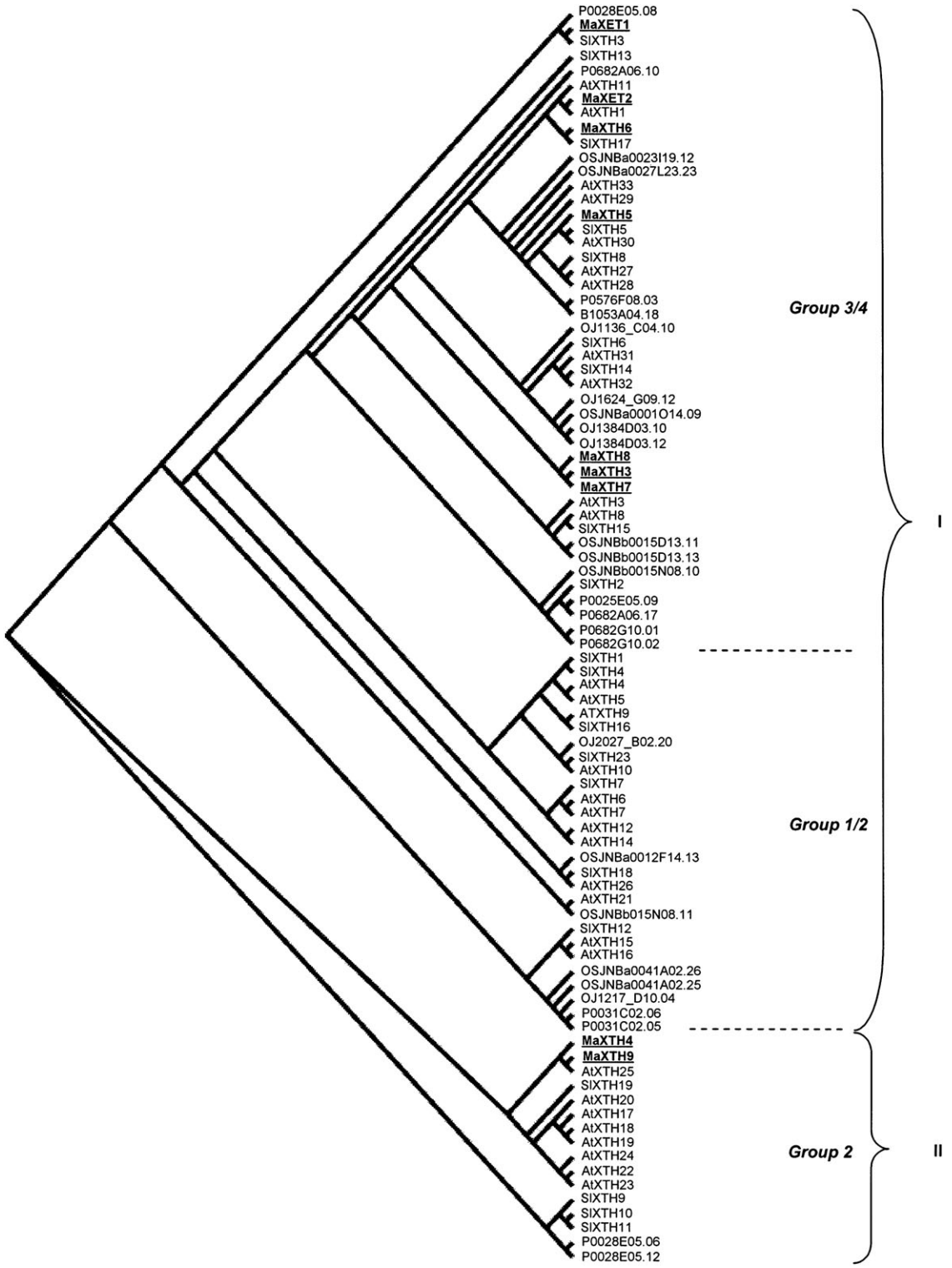

Fig. 2. Phylogenetic alignment of banana, tomato, *Arabidopsis*, and rice xyloglucan endotransglycosylase/hydrolases (XTHs). The regions in common between the XTH polypeptide sequences of banana (*MaXTHn* and *MaXETn*), tomato (*SlXTHn*), *Arabidopsis* (*AtXTHn*), and rice were used to contruct a phylogenetic tree using the ClustalX program. The resulting slanted cladogram was visualized with the TREEVIEW program (Page, 1996). The sequences were grouped into two distinct groups (I–II). The numbers 1 to 4 indicate the four families groups identified in tomato and *Arabidopsis* and applied to a broad range of plant species (Nishitani, 1997; Campbell and Braam, 1999; Rose *et al.*, 2002; Saladié *et al.*, 2006). The tomato and *Arabidopsis thaliana* XTH sequences used in this study are available online at http://labs.plantbio.cornell.edu/XTH/genes.htm. The rice XTHs were retrieved from the Rice genome research program (http://RiceGAAS.dna.affrc.go.jp, 7 February 2009) and are indicated in the cladogram by their gene number assigned by RiceGAAS. Banana *MaXET1* and *MaXET2* have been registered in GenBank under accession numbers EF103137 and EF103136, respectively.

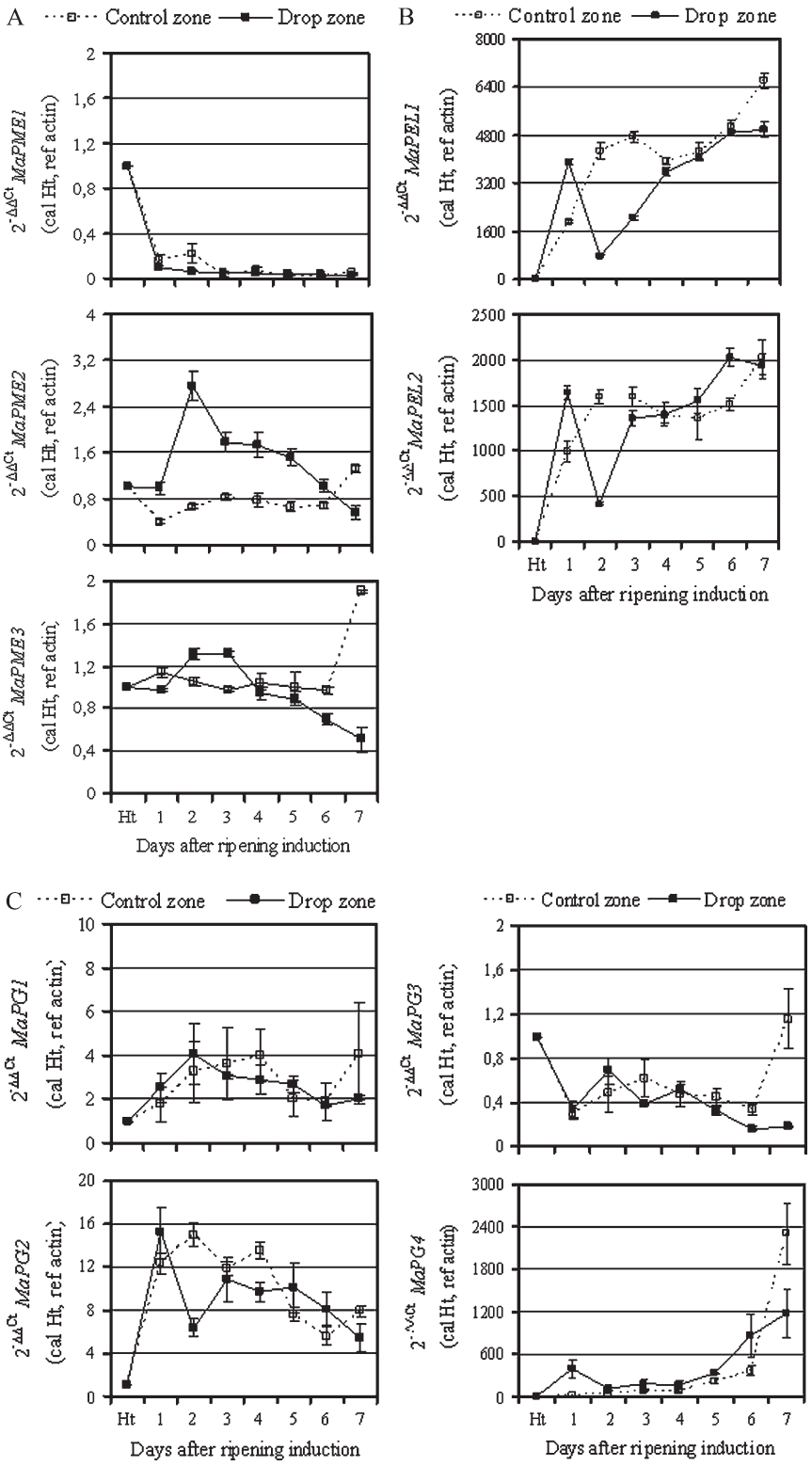

Fig. 3. Expression of banana pectolytic genes in banana peel tissue from the control and drop zones. Quantitative real-time PCR (RT-qPCR) was used to analyse mRNA accumulation of banana pectolytic genes, including pectin methylesterase (A), pectate lyase (B), and polygalacturonase (C) in peel tissue sampled from the middle area (control zone) and rupture area (drop zone). The *y*-axis represents the relative fold difference in mRNA level and was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001) with actin as reference. The mRNA fold difference was relative to that of fruit taken at harvest time (Ht) used as calibrator. Each data point is the mean of values obtained from qPCR reactions performed in triplicate on one cDNA sample. Each sample was prepared from four fruits from three replicate bunches. Two biological experiments were performed and they gave similar results. Vertical bars indicate standard deviation (SD). When no bar is shown, the SD is smaller than the symbol.

constant during the first 6 d after ripening induction. A slight increase was only observed in overripe fruit taken 7 d after ripening induction. In the drop zone and from day 1 after ripening induction to day 6, the *MaPME2* mRNA level was approximately 2-fold increased on average, compared with the control zone, with a maximum 3-fold increase observed 2 d after ripening induction and a minimum observed in overripe fruit taken 7 d after ripening induction. During ripening, no marked changes were observed on the *MaPME3* mRNA level in both control and drop zones, except at the late ripening stage when *MaPME3* mRNA accumulated approximately 2-fold more in the control zone than in the drop zone.

From day 1 to day 7 after ripening induction, both *MaPEL1* and *MaPEL2* mRNA levels increased drastically and continuously in the control zone (Fig. 3B). One day after ripening induction, *MaPEL1* and *MaPEL2* mRNA were 2-fold more expressed in the drop zone than in the control zone. The time-course of both *MaPEL1* and *MaPEL2* mRNA presented a transient decrease (4-fold) in the drop zone at day 2 after ripening induction. Thereafter, transcripts of both *MaPEL* genes increased to reach a level comparable with that of the control zone at day 3 for *MaPEL2* and 4 for *MaPEL1*, respectively.

Concerning polygalacturonase genes, the four cDNAs previously isolated in banana present, at nucleotide level, a high percentage of identity. *MaPG1* and *MaPG2* were 98% identical, although *MaPG1* has an additional 160 bp. *MaPG3* and *MaPG4* were over 90% identical while *MaPG2* and *MaPG3* were only 30% identical (Asif and Nath, 2005). Despite this, a set of qPCR primers was designed, as specific as possible, for each *MaPG* gene. For *MaPG1* that spans the entire *MaPG2* cDNA, primers were designed within the additional 160 bp in the 3′-UTR. For *MaPG2*, there was a failure to identify a divergent region with *MaPG1* to design a specific *MaPG2* primer. Nevertheless, *MaPG1* and *MaPG2* displayed quite different accumulation patterns, suggesting that putative cross amplification of the *MaPG1* gene with the *MaPG2* primer set would not have interfered during the gene expression analysis. No marked changes were observed on *MaPG1* gene expression during fruit ripening in both the control and drop zones (Fig. 3C). In the control zone, the time-course of *MaPG2* mRNA accumulation increased during ripening from day 1 to day 4 after ripening induction and decreased thereafter. A similar pattern was observed for *MaPG2* in the drop zone, except for a marked but transient decrease (2-fold) observed at day 2 after ripening induction. The mRNA level of the *MaPG3* gene decreased during ripening in the same manner in both the control and drop zones until day 6 after ripening induction. In overripe fruit taken at day 7, this level was approximately 10-fold higher in the control zone than in the drop zone. However, this level remained comparable to that of green fruit sampled at harvest. Finally, concerning *MaPG4*, the corresponding transcript was drastically induced in the control zone during the late ripening stages, namely 5, 6, and 7 d after ripening induction. In the drop zone, two peaks of *MaPG4* mRNA accumulation were observed. The first one occurred 1 d after ripening induction, with approximately 400-fold more expression in the drop zone than in the control zone, and the second one, during the late ripening stage, began at day 5 after ripening induction and reached its maximum at day 7, but at a 2-fold lower level than in the control zone.

Expression of MaXTH *genes in peel tissues from control and drop zones during banana fruit ripening*

In addition to the two XET genes, namely *MaXET1* and *MaXET2*, previously isolated from banana fruit (Lu *et al.*, 2004), seven other cDNA sequences were isolated in this study. In accordance with the nomenclature proposed by Rose *et al.* (2002), these cDNAs were named *MaXTH3* to *MaXTH9*. Specific primers designed within the 3′-UTR regions of each *XET* and *XTH* genes were used to examine the corresponding gene expression throughout qPCR analysis. Figure 4 shows the expression pattern in the drop and control zones of the nine *XTH* genes during banana fruit ripening. Except for *MaXTH4*, all other *XTHs* examined in this study were induced at different magnitudes in banana peel tissue, in the control and drop zones. In the control zone, *MaXET1* mRNA was transiently induced during ripening with maximum expression observed 4 d after ripening induction; in line with previous Northern blot data reported in banana (Lu *et al.*, 2004). Four *XTH* genes (*MaXET1*, *MaXTH6*, *MaXTH8*, and *MaXTH9*) were highly induced at the transcriptional level in the drop zone as compared to the control zone. However, this induction was transient, with a maximum of mRNA accumulation observed 3–4 d after ripening induction. *MaXTH9* was the most highly and transiently expressed as compared to the four others, while *MaXTH8* expression decreased slowly during the late ripening stage. By contrast, two *XTH* genes (*MaXTH3* and *MaXTH5*) were more highly and differentially expressed in the control zone than in the drop zone. *MaXTH3* was transiently induced in the control zone with a maximum observed 3 d after ripening induction, while in the drop zone no marked change was observed during post-harvest ripening. *MaXTH5* was less expressed in the drop zone compared to the control zone. In this area, *MaXTH5* presented two peaks of expression at days 3 and 7 after ripening, respectively. In the control zone, *MaXET2* and *MaXTH7* mRNA accumulation peaked twice at days 2 and 7, and at days 2 and 4 after ripening induction, respectively. In the drop zone, a high mRNA accumulation of *MaXET2* and *MaXTH7* genes was transient at days 4 and 3 after ripening induction, respectively. At this ripening stage, both genes were highly expressed in the drop zone compared to the control.

Expression of MaEXP *genes in peel tissues from control- and drop zones, during banana fruit ripening*

Based on five sequences of expansin genes already isolated from banana fruit (Trivedi and Nath, 2004; Sane *et al.*, 2007), specific primers were designed from the

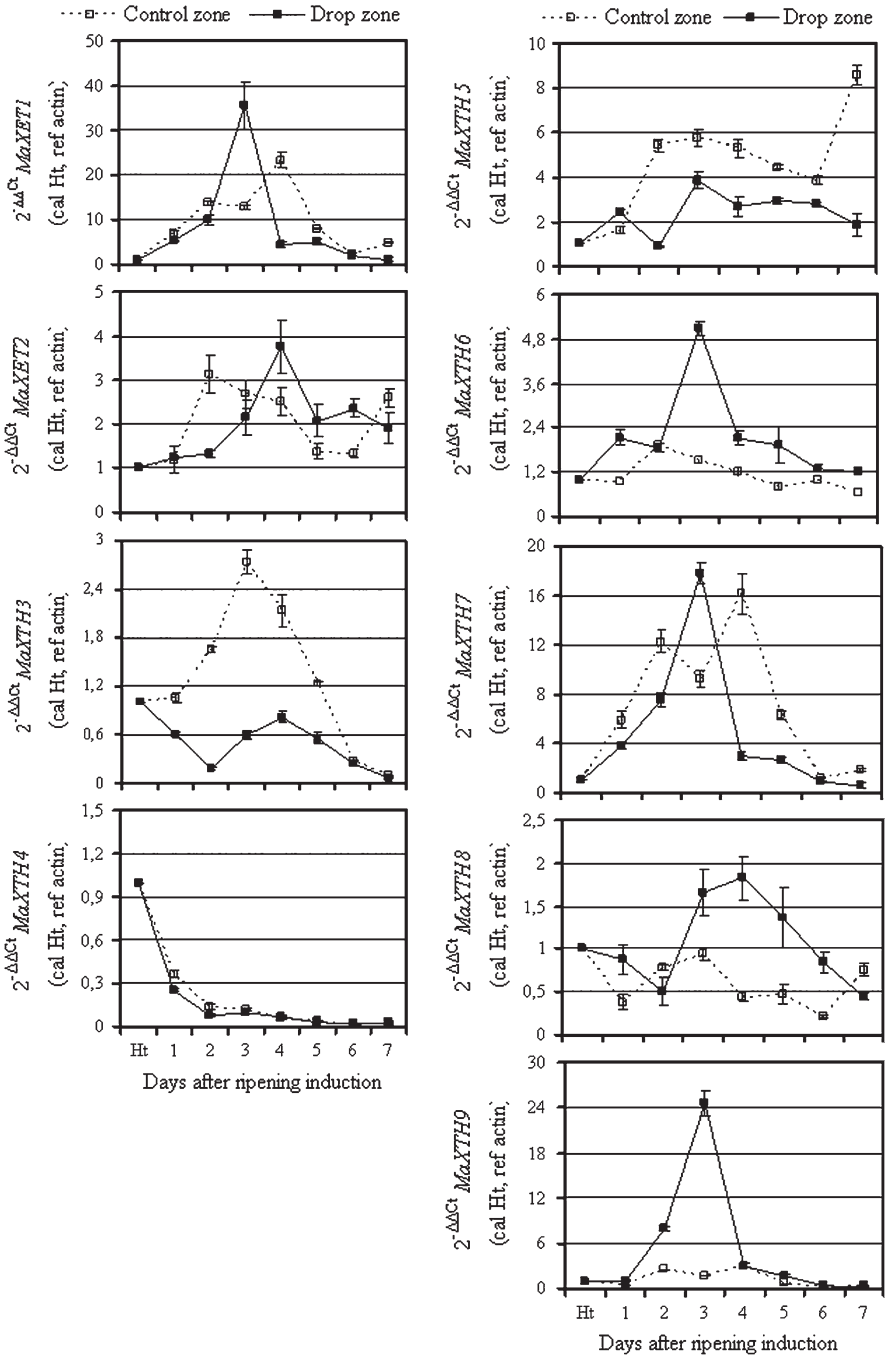

Fig. 4. Expression of banana *XTH* genes in banana peel tissue from the control and drop zones. Quantitative real-time PCR (RT-qPCR) was used to analyse the mRNA accumulation of banana xyloglucan transglycosylase/hydrolase genes in peel tissue sampled from the middle area (control zone) and rupture area (drop zone). The *y*-axis represents the relative fold difference in mRNA level and was calculated using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001), with actin as reference. The mRNA fold difference was relative to that of fruit taken at harvest time (Ht) used as calibrator. Each data point is the mean of values obtained from qPCR reactions performed in triplicate on one cDNA sample. Each sample was prepared from four fruits from three replicate bunches. Two biological experiments were performed and they gave similar results. Vertical bars indicate standard deviation (SD). When no bar is shown, the SD is smaller than the symbol.

corresponding 3′-UTR regions and used to examine expansin gene expression in both the control and drop zones (Fig. 5). Except for *MaEXP3*, from which there was a failure to get a single PCR product, the four other expansin genes were differentially expressed in control and drop zone tissues. By contrast with those of *MaEXP2*, mRNAs of *MaEXP1*, *MaEXP4*, and *MaEXP5* accumulated to a greater extent in the drop zone than in the control zone. *MaEXP2* mRNA presented a different accumulation pattern in both the control and drop zones as compared to the three other

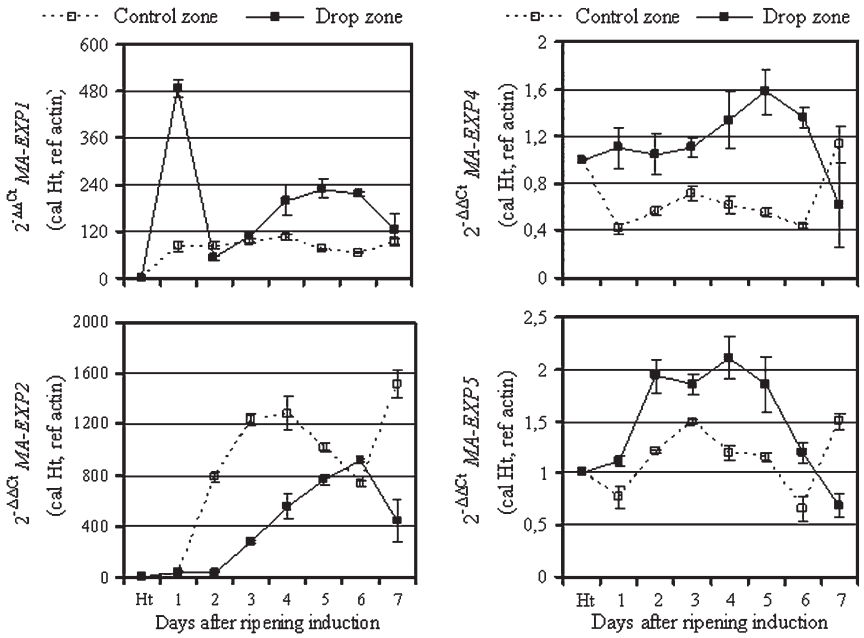

Fig. 5. Expression of expansin genes in banana peel tissue from the control and drop zones. Quantitative real-time PCR (RT-qPCR) was used to analyse the mRNA accumulation of banana expansin genes in peel tissue sampled from the middle area (control zone) and rupture area (drop zone). The *y*-axis represents the relative fold difference in mRNA level and was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001) with actin as reference. The mRNA fold difference was relative to that of fruit taken at harvest time (Ht) used as calibrator. Each data point is the mean of values obtained from qPCR reactions performed in triplicate on one cDNA sample. Each sample was prepared from four fruits originated from three replicate bunches. Two biological experiments were performed and they gave similar results. Vertical bars indicate standard deviation (SD). When no bar is shown, the SD is smaller than the symbol.

expansin genes analysed in this study. In the control zone, its mRNA began to accumulate 1 d after ripening induction and increased drastically (approximately 20–34-fold) until day 4 after ripening induction, when it peaked. Then this level slightly decreased at days 5 and 6, before increasing (2-fold) at day 7 after ripening induction. In the drop zone, *MaEXP2* mRNA accumulated continuously from days 1 to 6 after ripening induction, where it peaked before decreasing at day 7. Compared to the pattern in the control zone, *MaEXP2* was on average 1.3–20-fold less expressed in the drop zone during post-harvest ripening, except at day 6 where this expression was comparable in the two tissues. *MaEXP1* mRNA sharply accumulated (480-fold) 1 d after ripening induction. This level decreased at day 2 to reach a level comparable to that of the control zone before increasing transiently, on average 2-fold more, from days 4–6. At day 7, the *MaEXP1* mRNA level had reduced to a level comparable to that of the control zone. A marked increase in the *MaEXP4* mRNA level was observed during the late ripening stages, namely 4, 5, and 6 d after ripening induction before decreasing approximately 3-fold at day 7 after ripening induction. Compared to the control zone and except at the last ripening stage (day 7), *MaEXP4* mRNA accumulated, on average, 2-fold more in the drop zone after ripening, even during the first ripening stages (1–3 d after ripening induction), while this level was constant in the drop zone. In the drop zone, *MaEXP5* gene expression was transiently induced until day 4 after ripening induction, where it peaked before decreasing during the last three ripening stages. The same pattern was observed in the control zone, except that (i) peak mRNA accumulation was observed 3 d after ripening induction, and (ii) with a 2-fold increase at the last ripening stage (day 7). Compared to the control zone, the *MaEXP5* mRNA level was higher in the drop zone except at the last ripening stage.

Discussion

In the work described here, changes in the expression of various CWMGs (cell wall modifying genes) were examined in relationship with the banana finger drop process. This study was primarily aimed at identifying genes whose expression in the drop zone could be linked to the cell wall disassembly and property changes observed during banana ripening and finger drop by Imsabai *et al.* (2006) and Saengpook *et al.* (2007).

Current evidence suggests that cellulose disassembly is not a significant contributor to cell wall disassembly during fruit ripening (Fisher and Bennett, 1991; Newman and Redgwell, 2002; Bennett and Labavitch, 2008). Therefore, our molecular studies were focused on pectolytic and hemicellulosic genes and those involved in physical cell wall properties. Taken together, our data indicated that cell wall changes that occur in the finger drop area involve major cell wall components, including xyloglucan and pectin, and also cell wall physical properties, namely cell wall loosening mediated by expansin. Overall, major changes in cell wall

modifying gene expression occurred 1–4 d 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 those of xyloglucan genes, mainly during days 3–4. Moreover, within each mechanism, there was also sequential induction of the different genes involved.

The results of a comparative analysis of CWMG expression in drop and control zones allows the selection of candidate genes involved in the finger drop process. Assuming that the activity of CWMGs examined in this study such as PEL or PG are mainly regulated at the transcriptional level in banana (Pathak and Sanwal, 1998; Marin-Rodriguez *et al.*, 2003; Lohani *et al.*, 2004), it is considered that CWMGs expressed more in the drop zone than in the control zone were candidates.

A positive correlation was obtained between the banana cultivar prone to finger drop and PEL activity (Imsabai *et al.*, 2006). Both *MaPEL1* and *MaPEL2*, whose mRNA levels increased by 2-fold and 1.6-fold in the drop zone, possibly induced this increased PEL activity in the drop zone. Therefore, both *MaPEL1* and *MaPEL2* genes could be considered as candidates. Imsabai *et al.* (2006) reported that no clear change in PG activity was correlated with the finger drop process, and that PME activity does not account for the breakage of pectin associated with the finger drop process. Despite this, *MaPME2* and *MaPG4* could also be considered as putative candidates of finger drop based on the marked change in their mRNA accumulation in the drop zone relative to the control zone. The discrepancy between PG and PME gene expression and activities could be explained by post-transcriptional regulation of the activation of enzyme activity or subcellular localization (targeting cell walls). It is also possible that the PG and PME activities reported by Imsabai *et al.* (2006) might represent global activities that do not reflect a putative change in the activity of the different PG and PME isoforms. Note that the PG enzyme activity measured during banana fruit ripening suggested that more than the three identified isoforms might exist in fruit (Pathak and Sanwal, 1998; Pathak *et al.*, 2000). For other cell wall components, *MaEXP1*, *MaEXP4*, and *MaEXP5* genes appeared to be the main candidates involved in cell wall loosening related to finger drop, with *MaEXP1* being the main one, while *MaXET1*, *MaXET2*, *MaXTH6*, *MaXTH7*, *MaXTH8*, and *MaXTH9* appeared to be candidates for xyloglucan metabolism.

XTH proteins can display two distinct enzymatic activities, including transglycosylase enzymatic (XET) activity leading to xyloglycan chain synthesis or xyloglucan hydrolase activity (XEH) resulting in their degradation. These two activities are not correlated with the protein structure and subsequently with the XTH protein organization (Rose *et al.*, 2002; Fry 2005; Saladié *et al.*, 2006). On the other hand, the involvement of cell wall synthesis, including the xyloglucan component, throughout ripening and mainly between mature green and turning stages prior to perceptible softening, have been reported in tomato, i.e. another fleshy fruit like banana (Mitcham *et al.*, 1989, 1991; Greve and Labavitch; 1991; Huysamer *et al.*, 1997; Rose and Bennett, 1999). Because the XTH gene expression and phylogenetic data reported in this study do not presume the activity and the function of corresponding proteins, the involvement of both xyloglucan synthesis and degradation mediated by XTH proteins in the finger drop process cannot therefore be excluded.

Cell wall modification that occurs during the ripening process is complex. Although the data reported here highlight the involvement of the main cell wall modification enzymes, including PG, PEL, XTHs, and EXP, the possibility cannot be overlooked that, other secondary but important enzymes (β-galactosidase or α-L-arabinofuranosidase) or non-enzymatic mechanisms such as cell wall solubilization (Dumville and Fry, 2003; Zhuang *et al.*, 2006, 2007) could be involved in the finger drop process.

Ethylene production and fruit softening are two main features that characterize the ripening process of climacteric and fleshy fruit like banana. Our data suggested that finger drop could be related to these two processes. Indeed, finger drop implies transcriptional changes in genes that are also involved in fruit softening. However, some genes seem to be specific to softening (*MaXTH5*, *MaXTH3*, and *MaEXP2*), finger drop (*MaPME2*, *MaEXP1*, *MaEXP4*, and *MaEXP5*, *MaXTH6*, *MaXTH8*, and *MaXTH9*), or both (*MaPG4*, *MaPEL1*, and *MaPEL2*; *MaXET1*, *MaXET2*, and *MaXTH7*). Moreover, the two processes seem to be quite distinct in this variety, as shown by the type of genes involved and their sequential induction, with finger drop being the main induced process followed by softening. Regarding ethylene, our data showed that Cavendish banana fruit developed finger drop mainly during the early stage of fruit ripening (days 1–4), a period corresponding to a burst of ethylene production. Concomitantly, a high level of transcription of some ripening- and ethylene-dependent genes like *MaEXP1* and *MaPEL*s (Pua *et al.*, 2001; Trivedi and Nath, 2004) was also observed. It would be interesting to examine the relationship between ethylene production during ripening and the finger drop process. Does the specific induction of the banana finger drop process involve, at molecular level, ethylene production and/or require the ethylene transduction pathway or not?

In conclusion, the data reported here represent a substantial contribution towards understanding the post-harvest banana finger drop phenomenon at the molecular level. More specifically, the identification of several cell wall-modifying genes with expression profiles related to finger drop provided us with good candidates for functional studies in banana species. These candidate genes could be useful in molecular breeding schemes for banana improvement using cultivars differing in their proneness to develop finger drop and which are currently being assessed.

Supplementary data

Supplementary data can be found at *JXB* online.

Table S1. Sequences of primers used in this study.

Table S2. Different *cis*-regulatory elements found within the promoter of *MaPME2* and *MaPME3* genomic sequences.

Fig. S1. Multiple sequence alignment of polypeptide sequences of banana *XTH* genes.

Fig. S2. BAC localization and structure of banana *PME* genes and multiple sequence alignment of polypeptide sequences.

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