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RESEARCH PAPER

The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene

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Abstract

Six *MaMADS*-box genes have been cloned from the banana fruit cultivar *Grand Nain*. The similarity of these genes to tomato *LeRIN* is low and neither *MaMADS2* nor *MaMADS1* complement the tomato *rin* mutation. Nevertheless, the expression patterns, specifically in fruit and the induction during ripening and in response to ethylene and 1-MCP, suggest that some of these genes may participate in ripening. *MaMADS1*, *2*, and *3*, are highly expressed in fruit only, while the others are expressed in fruit as well as in other organs. Moreover, the suites of *MaMADS*-box genes and their temporal expression differ in peel and pulp during ripening. In the pulp, the increase in *MaMADS2*, *3*, *4*, and *5* expression preceded an increase in ethylene production, but coincides with the CO_2 peak. However, *MaMADS1* expression in pulp coincided with ethylene production, but a massive increase in its expression occurred late during ripening, together with a second wave in the expression of *MaMADS2*, *3,* and *4*. In the peel, on the other hand, an increase in expression of *MaMADS1*, *3,* and to a lesser degree also of *MaMADS4* and *2* coincided with an increase in ethylene production. Except *MaMADS3*, which was induced by ethylene in pulp and peel, only *MaMADS4*, and *5* in pulp and *MaMADS1* in peel were induced by ethylene. 1-MCP applied at the onset of the increase in ethylene production, increased the levels of *MaMADS4* and *MaMADS1* in pulp, while it decreased *MaMADS1*, *3*, *4*, and *5* in peel, suggesting that *MaMADS4* and *MaMADS1* are negatively controlled by ethylene at the onset of ethylene production only in pulp. Only *MaMADS2* is neither induced by ethylene nor by 1-MCP, and it is expressed mainly in pulp. Our results suggest that two independent ripening programs are employed in pulp and peel which involve the activation of mainly *MaMADS2*, *4*, and *5* and later on also *MaMADS1* in pulp, and mainly *MaMADS1*, and *3* in peel. Hence, our results are consistent with *MaMADS2*, a SEP3 homologue, acting in the pulp upstream of the increase in ethylene production similarly to LeMADS-RIN.

Key words: Developmental control, ethylene, 1-MCP, peel, pulp, ripening.

Introduction

Fruit ripening is a genetically controlled program requiring the co-ordination of fruit softening, colour change, aroma development, sugar accumulation, and a reduction in acid levels. This program engages developmental control components and in climacteric fruit, also components of the ethylene synthesis and response pathways (Theologis, 1992; Lelievre *et al.*, 1997; Alexander and Grierson, 2002; Adams-Phillips *et al.*, 2004; Barry and Giovannoni, 2007; Cara and Giovannoni, 2008; Pech *et al.*, 2008). Climacteric fruit exhibit a peak of respiration coinciding with an increase in ethylene production at the onset of the ripening process.

Recently, facilitated by the cloning of the genes mutated in several ripening-inhibited spontaneous tomato mutants, a cascade of transcription regulators acting upstream of the ethylene pathway has been revealed (Giovannoni, 2004, 2007). *MADS*-box genes play a major role in the molecular circuit of developmental regulation (Giovannoni, 2001, 2004; Vrebalov *et al.*, 2002; Vrebalov *et al.*, 2009; Itkin *et al.*, 2009). Type II MADS-box proteins, which constitute a group within the family of MADS-box genes, are characterized by the existence of M, I, K, and C domains. The DNA-binding domain (M), located at the N-terminus, and the K domain which is separated by 30 amino acids (aa) of the I domain, are highly homologous among various genes of the family (Theissen *et al.*, 2000; De Bodt *et al.*, 2003). The C region, on the other hand, is highly variable and it was implicated in transcriptional activation in higher order complex formation (Egea Cortines *et al.*, 1999; Garcia-Maroto *et al.*, 2003). Phylogenetic analysis revealed that type II MADS-box orthologues can be subdivided into distinct clades, and members of the same clade tend to have similar expression patterns (De Bodt *et al.*, 2003).

Several MADS-box paralogues have been identified in tomato fruit (Busi *et al.*, 2003; Giovannoni, 2007) and it was suggested that they may be involved in fruit development. The genes *TAG1*, *TAGL2*, *TAGL11*, *TAGL12*, *TAGL1*, *TDR6*, and *TDR4* were expressed during the first steps of tomato fleshy fruit development. Overexpression of *TAG1* resulted in fleshy expansion and ripening-like cell wall metabolism in sepals, indicating that it is responsible for fruit development (Pnueli *et al.*, 1994). The *TAGL2* protein, a *SEP* homologue, was found to interact with four MADS-box proteins, suggesting that MADS-box genes in the tomato fruit create heterodimers, possibly using the transactivation domain of *TAGL2* to activate transcription (Busi *et al.*, 2003). In addition, down-regulation of *TAGL2* (*TM29*), caused parthenocarpic fruit development, indicating that this gene may function as a negative regulator of fruit development (Ampomah-Dwamena *et al.*, 2002). *TDR4* belongs to the *SQUAMOSA* (*AP1*) clade, and its transcription is controlled, at least in part, by the SQUAMOSA Binding Protein *LeSPL-CNR* (*SQUAMOSA promoter binding protein-like-Colourless Non-Ripening*). The *LeSPL-CNR* gene is silenced by hypermethylation at its promoter in the *Cnr* mutant, causing a delay in fruit ripening (Manning *et al.*, 2006; Seymour *et al.*, 2008). It is still not yet clear if these additional MADS-box genes are involved in fruit ripening, and the functional analysis of these genes is underway in several laboratories. By contrast, the MADS-box gene, *LeRIN* was thoroughly investigated. Transgenic tomato plants under-expressing this gene had delayed ripening and a deletion of a segment from the C-terminus, which is the basis of the *rin* mutant, completely prevented ripening in the homozygous state (Vrebalov *et al.*, 2002). The gene is highly expressed during ripening (Vrebalov *et al.*, 2002) and most likely it controls ethylene production (Kitagawa *et al.*, 2005). Although fruit ripening was blocked in the *rin* mutant, it still responded to exogenous ethylene, indicating that the machinery responsible for ethylene response is still functional in these plants (Lincoln and Fischer, 1988; Giovannoni, 2001). A potential orthologue of *LeRIN*, *FvMADS9*, was isolated from strawberry, and was found to be expressed specifically in fruit (Vrebalov *et al.*, 2002).

Additional MADS-box genes were also discovered in other fruits; however, most of them were suggested to be involved in early fruit development. In apple, six *MdMADS* genes were classified to the *AP1* clade and one to the *AG* (Yao *et al.*, 1999), and they were found to be expressed during early fruit development. Mutation in apple *MdPI*, a MADS-box gene that is highly homologous to *PI*, known to be involved in identity determination of petals and stamen in *Arabidopsis*, was found to result in parthenocarpic fruit development (Yao *et al.*, 2001). In peach, two MADS-box genes similar to *TAG* and *TAGL1* have been cloned and their expression in tomato show that they might be responsible for fruit development (Tadiello *et al.*, 2009). In grapes, several isolated MADS-box genes were related to fruit development; *VvMADS1* and *VvMADS5* are homologous to *AG* and *SHP* (Boss *et al.*, 2001), *VvMADS2* and *VvMADS4* are related to *SEP*, and another one to *AGL13* gene (Boss *et al.*, 2001). Similar genes (*CanMADS*) were also isolated from hot pepper (Sung *et al.*, 2001) and hazelnut (Rigola *et al.*, 1998). MADS-box genes have also been isolated from Chinese pear and there was no difference in their expression in climacteric and non-climacteric cultivars (Yamane *et al.*, 2007). Whether or not these genes may be specifically involved in fruit ripening, remains to be determined.

Banana, like tomato, is a climacteric fruit, which is characterized by an increase in respiration and a burst in ethylene production occurring at the onset of ripening. However, in light of the fact that banana is a monocot, it is still not clear if this fruit uses similar components to those of the tomato eudicot for controlling ripening. In addition, banana ripening differs from that of tomato also because it has pulp and peel, which, based on their altered pattern of ethylene synthesis, suggests that their ripening programmes differ (Clendennen *et al.*, 1997). Ethylene production in peel starts after its production in the pulp and its initiation is dependent on pulp ethylene (Dominguez and Vendrell, 1993). So far, components related to the ethylene control of ripening have been cloned in banana. The expression of several ACC oxidase and synthase genes were correlated with increased ethylene levels (Clendennen *et al.*, 1997; Liu *et al.*, 1999; Pathak *et al.*, 2003). An ethylene receptor (Wu *et al.*, 1999), a *CTR1* orthologue (Clendennen *et al.*, 1997) and four EIN3-like genes were isolated (Mbeguié-A-Mbeguié *et al.*, 2008). Coinciding with the biochemical changes observed during banana fruit ripening, differentially-expressed genes were isolated from the pulp (Clendennen and May, 1997; Medina-Suarez *et al.*, 1997) and peel (Drury *et al.*, 1999; Liu *et al.*, 2002) of banana fruit after the initiation of the ripening process. MADS-box genes have been isolated from a Brazilian cultivar and from the cultivars Pisang bregnan and Nanicao, and have been deposited in GenBank (Liu *et al.*, 2009). The expression of a MADS-box gene (GenBank accession 941800 from Pisang)

increased after propylene application and this increase was reduced following 1-MCP treatment (Inaba *et al.*, 2007). Also the expression of MADS-box gene cloned from the Brazilian cultivar (GenBank accession DQ060444) was correlated with an increase in ethylene production (Liu *et al.*, 2009). However, so far there has been no comprehensive study of the *MaMADS*-box genes expressed in banana fruit. In addition, the interaction between ethylene and the expression of fruit *MaMADS*-box genes has not been fully studied.

In this study, six full-length MADS-box genes from banana (*Grand Nain*) have been cloned and characterized, and the ability of two of these genes to complement the *rin* mutation in tomato has been examined. In addition to their expression in various banana organs and during ripening in peel and puld, the interactions between ethylene and the expression induction of these genes have been determined.

Materials and methods

Plant materials and treatments

Banana (*Musa acuminata* AAA Cavendish subgroup, Grand Nain) grown along the Mediterranean shore in Israel during the winter months, were used in this study. Several stages during banana ripening were identified based on the details described in INIBAP (International Network for Improvement of Banana and Plantains: www.inibap.org): from the first stage of green banana fruit through the third stage of yellow banana with green edges until the seventh stage when brown spots are apparent. Fingers from the upper hand proximal to the trunk were taken from at least five bunches, separated, sprayed with 0.1% thiobendazole, and air-dried. They were packed in aerated polyethylene bags and stored at 20 °C. Samples were taken from pulp and peel, separately. In addition, other banana plant organs were used; bulb core (BC), root (R), pseudo stem (PS), young leaf (YL), male flower (MF), female flower (FF), bract leaf (BL), male flower ovary (MFO), and female flower ovary (FFO).

Ethylene treatment of 10 $\mu l\ l^{-1}$, based on preliminary experiments, was applied on the third day after harvest (DAH) for 18 h. 1-MCP treatments of 0.3 $\mu l\ l^{-1}$ was applied at the onset of an increase in ethylene production (usually on the 8th DAH) for 18 h and samples for the determination of ripening parameters were taken on consecutive days up to 17 DAH.

Characterization of fruit-ripening parameters

Fruit ripening was determined using the following parameters: peel colour, fruit firmness, and carbon dioxide (CO_2) and ethylene emission (C_2H_4). Peel colour from the surface area of the individual upper banana fingers was determined using Minolta CR-300 (Minolta Corporation, New Jersey, USA) and the results are expressed as hue angle (°). Firmness was measured using a Chatillon Force tester (Ametek Inc., Florida, USA), and results are expressed in Newton (N).

Carbon dioxide and ethylene production were determined by sealing a banana finger in a 2 L sealed glass jar at 20 °C for 1 h. Samples were withdrawn from the sealed jars using gas-tight syringes. Carbon dioxide concentrations were determined by a Packard 7500 gas chromatograph (Packard, IL, USA) with a thermal conductivity detector and a CTR-I packed column using helium as a carrier gas. Ethylene concentration was determined with Varian 3300 gas chromatograph equipped with a flame ionization detector and a C-5000 packed column using nitrogen as the carrier gas.

Cloning of MADS box genes

Total RNA was extracted from separated banana peel and pulp and cut into 1 g tissue and frozen in –80 °C. The frozen tissue was pulverized under liquid nitrogen and 100 mg was used with the Spectrum Plant RNA Kit (Sigma, UK). Extraction was done as described (http://www.sigmaaldrich.com/sigma/bulletin/STRN250bul.pdf). The extracted RNA was digested and cleaned with the TURBO DNase kit according to the protocol described at the site (http://www.ambion.com/techlib/prot/bp_1907.pdf). RNA concentrations ranging between 50–350 $\mu g\ \mu l^{-1}$ and a ratio 1.8–2.0 (of absorbance at 260/280) was obtained. cDNA was prepared using the Verso™ cDNA kit (Thermo Fisher Scientific Inc., USA). Cloning was performed on a mixture of cDNA from peel and pulp of different developmental stages.

The *MaMADS1* fragment was cloned by screening a ripe banana pulp cDNA library at low stringency with a full-length probe of *LeMADS-RIN*. In addition, several fragments of MADS-box genes from banana fruit of various cultivars have been deposited in GenBank http://www.ncbi.nlm.nih.gov/BLAST: a fragment of 378 nucleotides of the cultivar Nanicao (AY463009: corresponding to *MaMADS2*) and a sequence of 888 nucleotides from the Brazilian cultivar (DQ060444: corresponding to *MaMADS5*) and three sequences from the Pisang cultivar, one of 931 nucleotides (AY941799: corresponding to *MaMADS3*), a sequence of 944 nucleotides (AY941800: corresponding to *MaMADS4*), and a sequence of 615 nucleotides (AY941798: corresponding to *MaMADS6*). Aided by these sequences, a full-length of *MaMADS* genes from the cultivar *Grand Nain* fruit have been cloned by successive reactions using the primers described in Supplementary Table 1 at *JXB* online. For each of the genes, the primers described in reaction A were used to clone an initial fragment; in reaction B, they were used for completing the 3′ end, and in reaction C, they were used for completing the 5′ end. Both reactions B and C used a RACE technology (5′/3′ RACE Kit 2nd generation, Roche Applied Science). The PCR amplifications were carried out in a Mastercycler gradient apparatus (Eppendorf, Germany) using 40 cycles of denaturing at 95 °C for 30 s, annealing at 52 °C for 60 s and elongation at 72 °C for 60 s. PCR product was further purified from the gel using QIAEX II gel extraction kit (Qiagen Ltd, UK) according to the manufacturer's instruction (http://www1.qiagen.com), and ligated to the pGEM-T® easy vector (Promega Corporation, Madison, USA). The ligation reaction was transformed into JM 100 competent *E. coli* cells (www.rbcbioscience.com) (RBC Bioscience).

BioEdit Sequence Alignment Editor software (v. 7.0.9) was used for the DNA alignment. The phylogenetic analysis was performed using the tools in the site (http://www.phylogeny.fr/version2_cgi/index.cgi), which enables this analysis by PhyML, a software implementing a new method for building phylogenies from DNA and protein sequences using maximum likelihood, and the tree was drawn by TreeDyn (Dereeper *et al.*, 2008). Primer Express software (v. 2.0) was used for design of primers for quantitative RT PCR (Q-RT-PCR) and for cloning (http://www.appliedbiosystems.com/support/apptech/#).

Expression analysis of MaMADS*-box genes*

Gene expression was determined by Quantitave RT PCR (Q-RT-PCR). The sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a ribosomal gene (AY821550 and EU433925, respectively) were used as a reference for equalizing the levels of RNA. Forward and reverse primers for the references genes are: 5′-GCAAGGATGCCCCAATGT-3′ and 5′-AGCAAGACAGTTGGTTGTGCAG-3′ for GAPDH, 5′-GCGACGCATCATTCAAATTTC-3′ and 5′-TCCGGAATCGAACCCTAATTC-3′ for the

ribosomal gene. Primers and cDNA concentrations used for the reactions were predetermined as described, to enable a linear and highly efficient response (http://www.abgene.com/downloads/article-SYBRoptimise.pdf). The reaction mixture contained forward and reverse primers (see Supplementary Table 2 at *JXB* online) and Power SYBR Green PCR Master mix (Applied Biosystems, USA) in a 20 µl total sample volume. Reactions were run in triplicate on a Rotor-Gene 3000 PCR machine (Corbett Life Research, Australia) using 35 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. The results represent one experiment out of at least two independent samplings, for which, usually two preparations of cDNA were examined. Data obtained were analysed with Rotor-Gene 6 software, and by the qBase quantification Software (http://medgen.ugent.be/qbase/). Data are expressed according to the ΔΔCT method. To enable presentation of all the different transcripts in response to either ethylene or 1-MCP, expression is expressed as a percentage increase of the lowest sample.

Complementation of rin *mutants*

Tomato plants used in complementation studies were homozygous for the *rin* mutation in cultivar Ailsa Craig and grown in greenhouses under standard conditions with 16/8 h day/night.

Full-length cDNAs of *MaMADS1* and *MaMADS2* were cloned into the plant transformation vector pBI121 and transformed into *rin*/*rin* tomato as described in Vrebalov *et al.* (2002). Kanamycin-resistant transformants were grown to maturity in the greenhouse and confirmed for transgene integration by DNA gel-blot analysis using the *npt*II gene (Kan resistance) as probe. Seeds were saved and all subsequent analyses for ripening complementation were performed on T_1 progeny which either contained or segregated out the transgene, as confirmed by DNA gel-blot analysis. A total of 11 and 17 independent transgenic lines were generated for *MaMADS1* and *MaMADS2*, respectively.

Results

Isolation of MADS-box genes from banana fruit

Six full-length transcripts were cloned from the banana fruit of the cultivar *Grand Nain* of the Cavendish subgroup (Fig. 1). Each one of them exhibited a difference in the amino acid (aa) sequence, when compared to the homologuos partial sequences from other other banana cultivars deposited in GenBank, (data not shown). Alignment of the putative aa of the six genes showed high similarity in the two regions: 1 to about 55 aa–M domain, and around 95 to about 170 aa–K domain (Fig. 1A). The genes were highly variable in their C domain. Phylogenetic analysis of these genes with MADS-box genes from various clades revealed that the genes *MaMADS1–4* belong the *SEP3* clade and indeed they contain the *SEP3* motif (Fig. 1B) (Malcomber and Kellogg, 2005). The genes *MaMADS2* and *4* show the highest similarity between them and the highest to *LeMADS-RIN* (Table 1). The genes *MaMADS5* and *MaMADS6* belong to the *AGAMOUS* (*AG*) and *PISTILLATA* (*PI*) clades, respectively. These genes indeed contain the respective domains (Fig. 1 C, D) (Skipper *et al.*, 2006). Comparing the C-terminus of all the banana genes to that of *LeMADS-RIN* from tomato showed lower homology to *Le-MADS-RIN* than when the whole gene is compared (Table 1). (Similarities are: 30% for *MaMADS1*, 35% for *MaMADS2*, 28% for *MaMADS3*, 32% for *MaMADS4*, 20% for *MaMADS5*, and 15% for the gene *MaMADS6*). The genes isolated have shown the highest homology to the corresponding genes: *MaMADS1*: 75% similarity to *AOM1* (AAQ83834 from *Asparagus officinalis*) (Caporali *et al.*, 2000), *MaMADS2* and *MaMADS4*: 88% and 84% similarity, respectively, to MADS-box (AAQ03226 from *Elaeis guineensis*) (Adam *et al.*, 2006), *MaMADS3*: 78% similarity to *AGL9a* (ABK35281from *Crocus sativus*). *MaMADS5*: 85% similarity to *MADS* (BAD83772 from *Asparagus virgatus*), *MaMADS6*: 88% similarity to *PISTILLATA*-like protein (*ABB92623 Alpinia oblongifolia*) (Gao *et al.*, 2006).

The expression of these genes in vegetative and reproductive tissues has been examined (Fig. 2). *MaMADS1–5* genes are highly expressed in either peel or pulp, however, *MaMADS4* and *5* are also highly expressed in other reproductive tissues. *MaMADS4* and *MaMADS5* are expressed in the female flower ovary (FF-O), and only *MaMADS4* is also expressed in the vegetative tissue pseudo stem (PS). The genes *MaMADS1* and *2* are highly expressed in peel and pulp, however, *MaMADS2* is also expressed in other tissues at lower levels. By contrast, *MaMADS6* is highly expressed in the male flower (MF), more than in fruit. This analysis shows that, in peel, *MaMADS1* and *3* are predominant during the climacteric stage, while, in pulp, *MaMADS2*, *4*, and *5* are most highly expressed.

MaMADS-*box genes expression in peel and pulp during normal ripening*

The *LeMADS-RIN* which was proven to be involved in fruit ripening, exhibited elevated expression at the onset of ripening (Vrebalov *et al.*, 2002). Therefore, the expression patterns of all the banana fruit *MaMADS* genes have been determined during ripening to examine which of the genes shows similar expression to that of *LeMADS-RIN* (Figs 4, 5). Production of ethylene and carbon dioxide in whole fruit and ripening parameters of colour and firmness have been determined following harvest (Fig. 3). Carbon dioxide climacteric production preceded the peak of ethylene, and it coincided with a reduction in firmness (Fig. 3A, B). Changes in colour (from green to yellow) appeared 4 d later and started following the ethylene peak (Figs. 3A, B).

The patterns of expression of the various genes in the peel and pulp were unique to the tissue examined. An increase in expression of most of the genes correlated with CO_2 production in the pulp and with ethylene production in the peel (Figs 4, 5), except the expression of *MaMADS6* in pulp which exhibited high expression during the green stage immediately after harvest and prior to any increase in ethylene or CO_2 production (Fig. 4). Expression of *MaMADS6* in peel was only slightly induced, but prior to the onset of the ethylene peak (Fig. 5). Also the expression of *MaMADS5* in peel was not increased in parallel to ethylene or CO_2 overproduction and was constant during the fruit ripening period (Fig. 5). The increased expression of *MaMADS2*, *3*, and *5* in pulp paralleled the increase in CO_2 production and that of *MaMADS4* followed. The

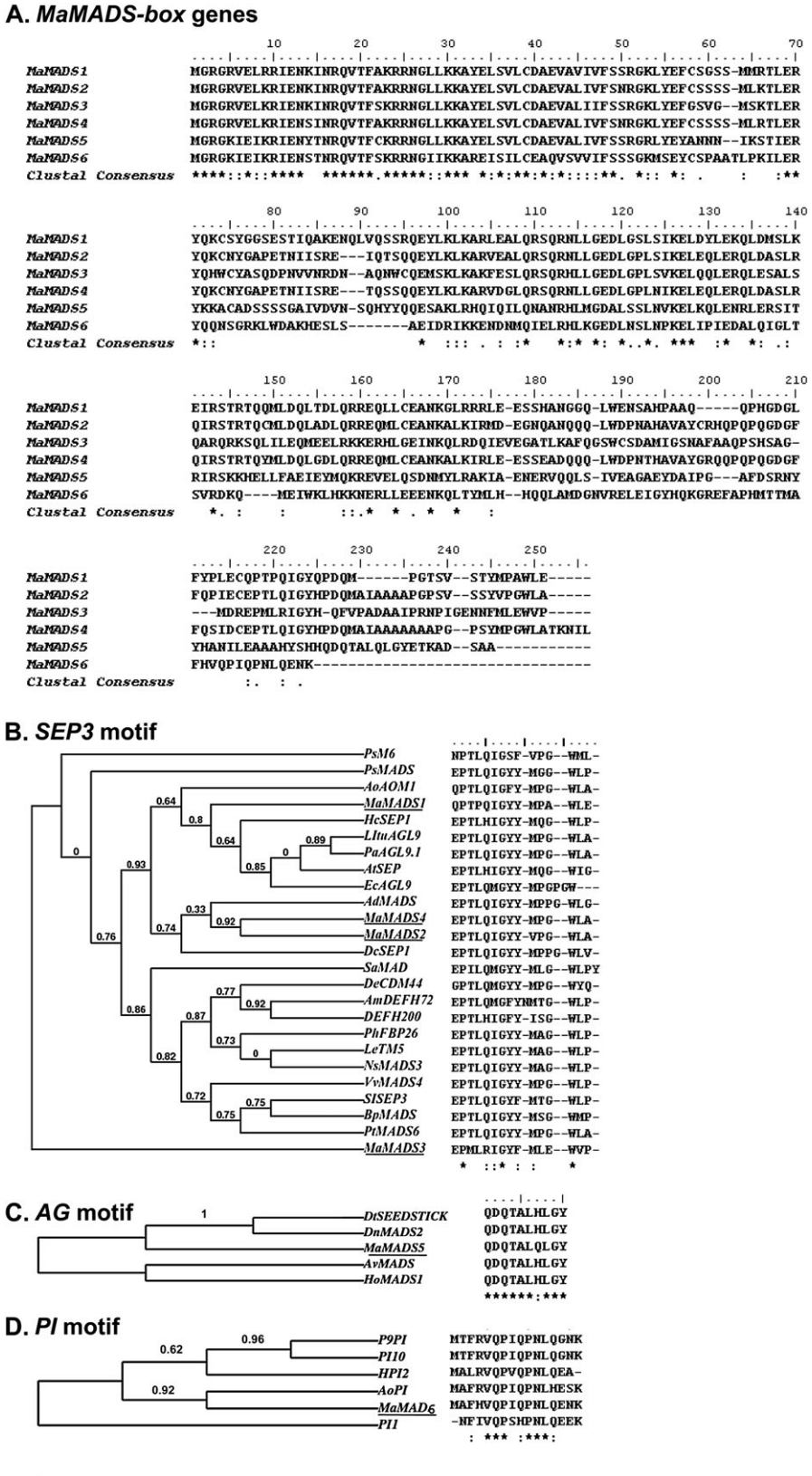

Fig. 1. Isolation of *MaMADS*-box genes from Cavendish banana (cultivar *Grand Nain*). (A) Putative proteins alignment of *MaMADS1–6* sequences isolated from banana fruit and the similarity among them. The gene sequences were deposited in http://www.ncbi.nlm .nih.gov/BLAST as *EU869307*, *EU869306*, *EU869308*, *EU869309*, *EU869310*, and *EU869311*, respectively. (B) Phylogenetic analysis of *MaMADS1–4* with other *SEP3* clade genes. The analysis was performed on the full-lengths of the individual genes. The *SEP3* motif for each of the genes is presented on the right. The genes included in this alignment are: *AoAOM1 (AAQ83834)*, *SlSEP3 (BAD10945.1)*, *LItuAGL9 (AAX15920.1)*, *PaAGL9.1 (AAX15923.1)*, *HcSEP1 (BAC80253.1)*, *PsM6 (AAX69068)*, *DcSEP1 (AAZ95252.1)*, *AdMADS*

Table 1. Comparison between the different *MaMADS*-box genes and *LeRIN*

Numbers indicate percentage homology at the amino acid level.

	MaMADS1	*MaMADS2*	*MaMADS3*	*MaMADS4*	*MaMADS5*	*MaMADS6*	*LeRIN*
MaMADS1		71	52	69	48	42	56
MaMADS2	71		54	88	41	37	62
MaMADS3	52	54		55	44	43	55
MaMADS4	69	88	55		41	40	60
MaMADS5	48	41	44	41		43	49
MaMADS6	42	37	43	40	43		43
LeRIN	56	62	55	60	49	43	

increase in *MaMADS1* expression in pulp, on the other hand, appeared at the onset of ethylene production and a major increase occurred late, after harvest, when the banana was completely ripe (stage 7). This induction was correlated with a second wave of increased expression in the genes *MaMADS2*, *3*, and *4* (Fig. 4), indicating that there are two stages of MaMADS-box participation in ripening.

In the peel, the increase in expression of the genes *MaMADS1*, *2*, *3*, and *4* was correlated with the increase in ethylene production and, while *MaMADS3* and 2 remained high, that of *MaMADS1* and *4* decreased gradually during ripening (Figs 3, 5). Most notably, is that the expression levels of *MaMADS2* in peel were lower than those in pulp (Figs 3, 4, 5).

Interactions between ethylene and MaMADS-*box gene expression*

Since most of the gene transcripts were induced in parallel with the onset of ripening, the interactions between ethylene and *MaMADS*-box genes expression has been studied following the application of ethylene or 1-MCP (Figs 6, 7). Ethylene application at the green stage increased endogenous ethylene production (data not shown). This treatment also increased *MaMADS3*, *4*, and *5* in the pulp and *MaMADS1* and *3* in the peel. However, this treatment did not increase the expression of *MaMADS2* either in the pulp or in the peel (Fig. 6).

1-MCP was applied prior to the ethylene peak (8 d following harvest in Fig. 7). This aimed to show a direct effect of ethylene on *MaMADS*-box gene expression at the onset of ripening. 1-MCP treatment on the 8th day reduced ethylene and CO_2 production (data not shown). This treatment decreased the expression of *MaMADS1*, *3*, *4*, and *5* in the peel, and, for *MaMADS1* and *3*, this represents a reciprocal image of the ethylene effect (Fig. 6). However, in the pulp, this treatment increased the expression levels of *MaMADS1* and *4*, suggesting that at this stage of ripening, these genes are under the negative control of ethylene. Also, this treatment, similar to that observed for ethylene, did not affect the expression levels of *MaMADS2* (Fig. 7).

Analysis of tomato plants expressing MaMADS1 *and* MaMADS2 *genes*

As a first step towards functional analysis, and since banana transformation is difficult and time-intensive, we transformed into the homozygous *rin* mutation of tomato cultivar Ailsa Craig, 35S-driven constructs of both *MaMADS1* and *MaMADS2*. A similar strategy was used to validate the function of the tomato *LeMADS-RIN* gene (Vrebalov *et al.*, 2002). *MaMADS2* was specifically selected for complementation of *rin* due to its closest sequence similarity to *LeMADS-RIN* and to the fact that it is not affected by ethylene and *MaMADS1* due to its high expression in peel and pulp.

Transgenic T_1 generation plants and non-transgenic siblings were confirmed for transgene overexpression in mature fruit (data not shown) and monitored for ripening parameters. No changes in ethylene, softening, and carotenoid accumulation in ripening were detected in transgenic *rin*/*rin* fruits (Fig. 8), nor were any abnormalities noted in floral or fruit development. Application of exogenous ethylene to these transgenic fruit also had no impact on their development.

Discussion

Gene structures and similarity to LeRIN

The full-length cDNAs of six MADS-box genes have been cloned from the banana fruit *Grand Nain* cultivar. The

(CAA48859.1), *SaMADSD (CAA69916.1)*, *PsMADS (CAA11258.1)*, *PhFBP26 (AAF19164.1)*, *NsMADS3 (AAD39034.1)*, *LeTM5 (AAP57413.1)*, *EcAGL9 (AAX15918.1)*, *DeCDM44 (AAO22982.1)*, *BpMADS (CAB95648.1)*, *DEFH200 (CAA64743.1)*, *PtMADS6 (AAO49811.1)*, *AtSEP (AAT46095.1)*, *VvMADS4 (AAM21344.1)*, *AmDEFH72 (CAA64742.1)*. (C) Phylogenetic analysis of *MaMADS5* with other *AG* genes. The existence of AG motifs for all the genes is shown. The genes included in this alignment are: *DtSEEDSTICK (AAY86365.1)*, *DnMADS2 (ABQ08574.1)*, *AvMADS (BAD83772.1)*, *HoMADS1 (AAF08830.2)*. (D) Phylogenetic analysis of *MaMADS6* with other *PI* genes. The existence of PI motifs for all the genes are shown. The genes included in this alignment are: *AoPI (ABB92623.1)*, *P9PI (AAV28175.1)*, *PI10 (AAV28490.1)*, *HPI2 (AAD22494.2)*, *PI1 (ABG90945.1)*. Lines underneath the genes mark the genes isolated from banana. Stars and dots indicate identity and similarity, respectively.

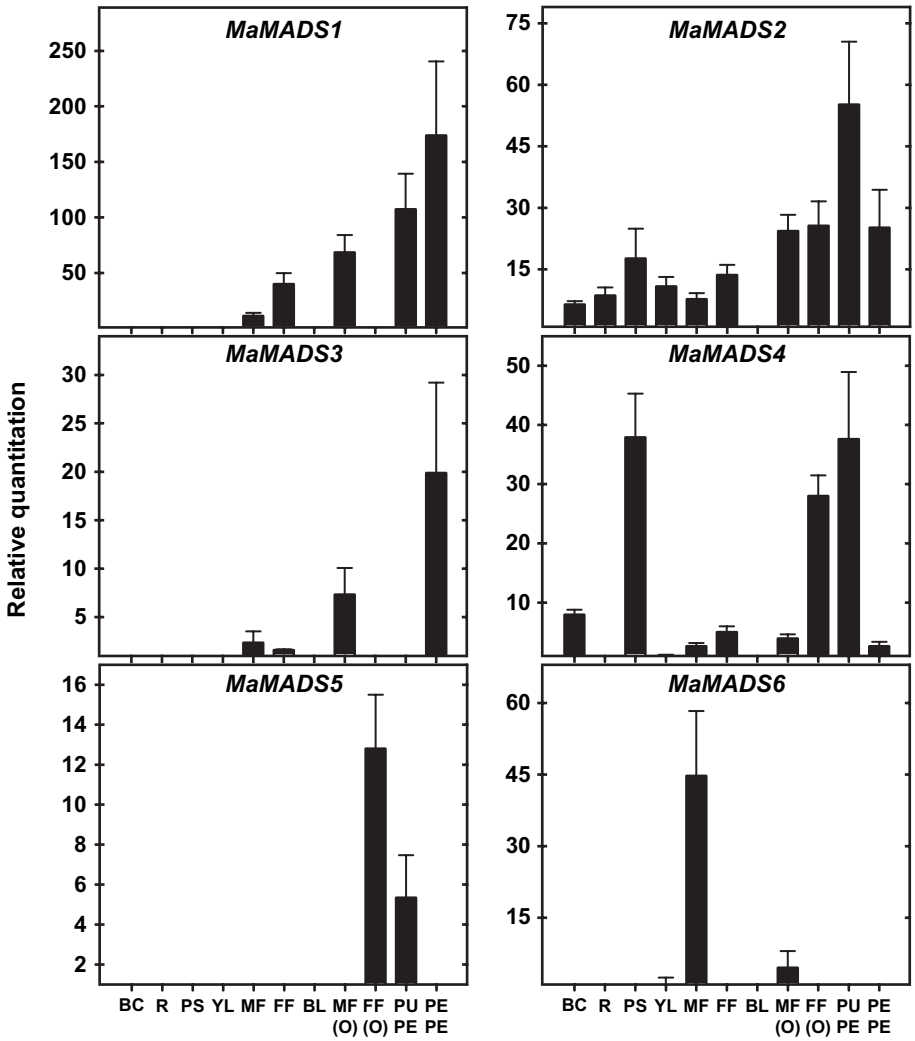

Fig. 2. Expression patterns of *MaMADS* genes in various plant organs. BC, bulb core; R, root; PS, pseudostem; YL, young leaf; MF, male flower; FF, female flower; BL, bract leaf; MF(O), male flower ovary; FF(O), female flower ovary; PUPE, pulp at climacteric peak; PEPE, peel at climacteric peak. Expression was determined by Q-RT- PCR as relative quantification. The specificity of the primers was determined for each of the genes (see Table 1, Materials and methods), and the expression of both ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference genes. Results are of a representative experiment, and are an average of three repetitions ±SD. The values of relative quantification obtained were 10^5 higher.

genes are highly divergent in their C-terminus. This is common among MADS-box genes even those arising from gene duplications. The differences between the genes may have arisen due to mutations that may lead to new functions (Maere *et al.*, 2005). The genes *MaMADS2* and *MaMADS4* show the highest similarity, even in their I region (Fig. 1A), which is responsible for partner selection (Garcia-Maroto *et al.*, 2003); however, the role of the I region in dimerization of these two genes has not been clarified yet. Phylogenetic analysis of these genes with MADS-box genes of various clades indicate that *MaMADS6* belongs to the *PI* clade, *MaMADS*5 to the *AG* clade, and *MaMADS1*, *2*, 3, and *4* belong to the *SEP3* clade and indeed these genes contain the typical motifs for each of the groups (Fig. 1) (Malcomber and Kellogg, 2005; Skipper *et al.*, 2006). Neither of the genes cloned were highly similar to the *LeMADS-RIN* which encodes a component of the developmental control of ripening and belongs to the *SEP4* clade. Nevertheless, the highest similarities of the *LeMADS-RIN* C-terminus are to that of *MaMADS2* and *4* genes, which are only 35% and 32%, respectively. It has been determined before that orthologues of *SEP4* are missing from non-core eudicots, monocots, and basal angiosperms (Malcomber and Kellogg, 2005), and might be the reason for not finding SEP4 homologue in banana.

Genes of these clades have been isolated in other fruits; the *PI* homologue was found to be mutated in an apple cultivar undergoing parthenocarpic development (Yao *et al.*, 2001) and *AG* genes, which are involved with carpel development, were isolated from apple fruit (Yao *et al.*, 1999), grapes (Boss *et al.*, 2001), peach (Tadiello *et al.*, 2009), and tomato (*TAG1*, *TAG11*, and *TAGL1*) (Busi *et al.*, 2003). *SEP* genes with high expression in fruit have also been identified in tomato (Giovannoni, 2007). The

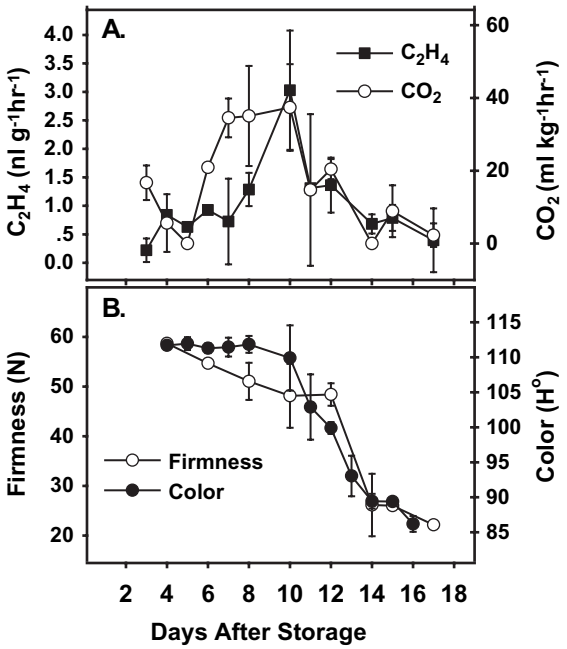

Fig. 3. Description of ethylene and carbon dioxide production (A) and ripening parameters (B) in whole fruit. The parameters were determined in banana from the upper hand immediately following harvest and at consecutive days during storage at 20 °C and 75% RH. Ripening parameters determined were peel colour (H° angle) and firmness (N).

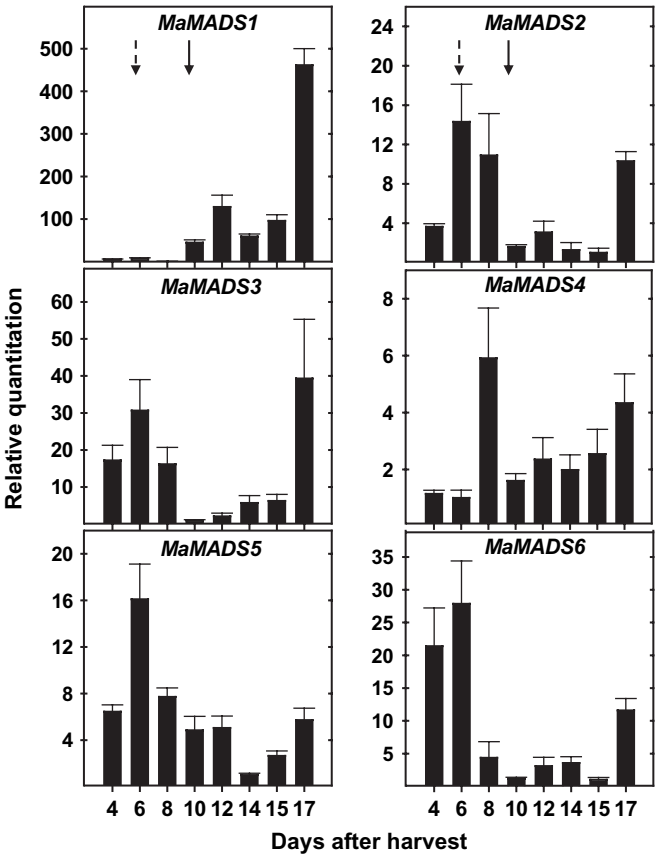

Fig. 4. Dynamic changes in *MaMADS*-box genes expression in pulp tissue during ripening. The expression was determined in fruit from the upper hand according to details described in Fig. 2. The increase in CO_2 production appeared 6 d after harvest (DAH) (broken arrow) and the ethylene peak in these fruits was detected by 10 DAH (full arrow). Results are of a representative experiment, and represent an average of three repetitions ±SD.

genes belonging to the *SEPALLATA* (*SEP*) clades often retain similar functional capacity (Zahn *et al.*, 2005) and they appear to contribute to the creation of multimeric complexes (Honma and Goto, 2001). In addition, MADS-box genes belonging to other clades have been cloned from tomato; like the gene *TDR6* which belongs to the *AP3* group, and *MC*, *TAGL2* and *TDR4* which belong to the *SQ/AP1* clade (Vrebalov *et al.*, 2002; Busi *et al.*, 2003). Studies in *Arabidopsis* and tomato revealed that *AG* together with *SEP* are responsible for carpel development (Seymour *et al.*, 2008; Vrebalov *et al.*, 2009) and it is possible that *MaMADS5* fulfil similar function in banana fruit development, possibly aided by *MaMADS6*, the *PI* homologue.

MADS-box genes have been cloned from a genome wide expression profile of another monocot, rice, and 75 MADS-box genes have been identified (Arora *et al.*, 2007). Protein alignment between the *MaMADS* and the rice MADS-box genes deposited in the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) revealed that *MaMADS1*, *MaMADS2*, and *MaMADS4* have about 70% homology to MADS-box transcription factor 8 (Os09g32948.1) and *MaMADS3* has about 73% homology to MADS-box transcription factor 6 (Os02g45770.1) all classified as *SEP3*. *MaMADS5* has about 66% homology to MADS-box transcription factor 3 (Os01g10504.3) classified as *AG*, while *MaMADS6* has a similar homology to MADS-box transcription factor 4 (Os05g34940.2) classified as *PI*. It is still not clear if these genes in rice have any function in ovary development and carpel maturation.

Expression patterns of MaMADS *genes and their possible involvement in ripening*

In many cases, MADS-box genes are not expressed exclusively in one tissue, but are recruited for different functions (Theissen *et al.*, 2000; Garcia-Maroto *et al.*, 2003; Immink *et al.*, 2003). Among the banana fruit MADS-box genes, only *MaMADS1–3* are expressed in fruit tissues at their highest levels, but the other genes are expressed at higher levels in other tissues (Fig. 2). The *MaMADS5* gene belonging to the *AG* clade, besides being expressed in fruit, was also expressed in the female flower ovary (Fig. 2), and *MaMADS6*, belonging to the *PI* clade, was expressed mainly in male flowers (Fig. 2), however, it was also expressed to some degree in the pulp and peel (Figs 4, 5), and the levels in pulp were even higher after harvest (Fig. 4). Also, among the *SEP3* genes, *MaMADS4* was expressed in the pseudo-stem as well as in the female flower ovary (Fig. 2). Indeed, genes belonging to the *SEP* clade have been found to be expressed mainly in inflorescences and act as redundant genes in flower development, and are even found to be expressed in vegetative tissue (Malcomber and Kellogg, 2005). In tomato too, some of the genes that were

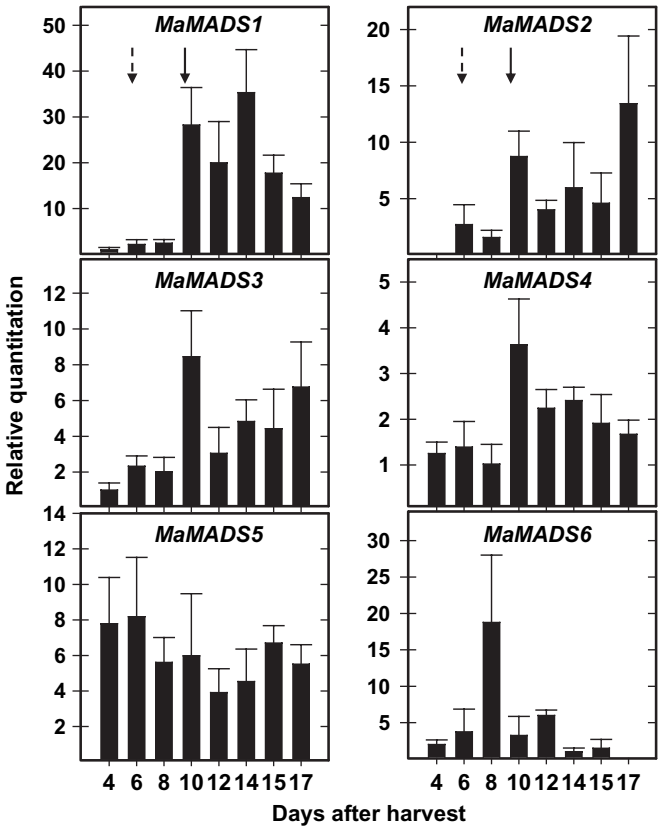

Fig. 5. Dynamic changes in *MaMADS*-box genes expression in peel tissue during ripening. The expression was determined in fruit from the upper hand according to details described in Fig. 2. An increase in CO_2 production appeared 6 d after harvest (DAH) (broken arrow) and the ethylene peak in these fruits was detected at 10 DAH (full arrow). Results are of a representative experiment, and represent an average of three repetitions ±SD.

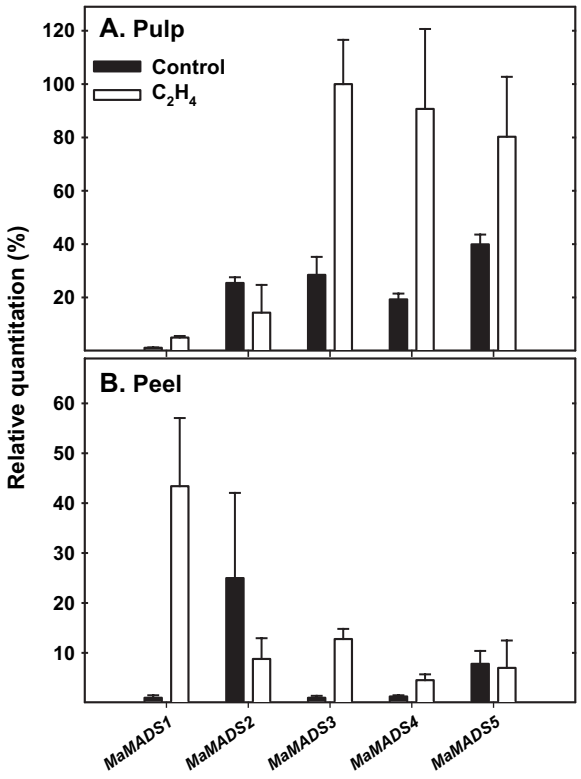

Fig. 6. Response to exogenous ethylene of *MaMADS*-box genes in peel and pulp. Banana fruits were treated with 10 μl l^{-1} ethylene 4 d after harvest for 18 h. The expression was determined for control and ethylene-treated samples relative to other samples during fruit ripening and the results of day 5 after harvest are presented.

expressed in fruit were also expressed in other organs. *TAGL12* was found not to be specific to fruit. *TDR6*, *TDR4*, and *TAG1* were also previously described to be involved in flower development (Busi *et al.*, 2003). In rice, another monocot, although MADS-box genes showed expression in reproductive tissue, they had a general tendency also to be expressed in vegetative tissues (Arora *et al.*, 2007).

The expression patterns during fruit ripening are very similar in the pulp for the genes *MaMADS2–5*, and in peel for *MaMADS1–4* (Figs 4, 5). Similar expression of genes, especially if they are from the same clade, had been suggested to show that they have a redundant function (Purugganan *et al.*, 1995; Theissen *et al.*, 2000; Immink *et al.*, 2003). These gene products may create heterodimers during the ripening process and different heterodimers may be created in the pulp and peel. Changes in the expression patterns of the various genes in the pulp and in peel during ripening (Figs 4, 5) suggest that the transcription complexes created during ripening are constantly changing.

Usually there is a parallel increase in ethylene and CO_2 at the onset of ripening in climacteric banana fruit (Domínguez and Vendrell, 1994) and in some studies it was reported that an increase in ethylene preceded the increases in respiration, when fruit were exposed to propylene (McMurchie *et al.*, 1972), or during natural ripening (Burg and Burg, 1965). In the current experiments, the CO_2 increase preceded the increase in ethylene by three days. It is interesting to note, that in several experiments that were performed in the winter months, the increase in CO_2 preceded that of ethylene by one day and up to three days, however, in the summer months the increase in ethylene and CO_2 appeared together (data not shown). The spatial separation between the increase in respiration and ethylene, which existed in these experiments, enabled us to determine that the increase in gene expression in the pulp of *MaMADS2–5* correlated with a CO_2 increase, and preceded ethylene increase. However, in experiments where ethylene and CO_2 increased concurrently, the increase in gene expression did not precede the ethylene peak (data not shown). In the peel, on the other hand, the increase in expression of *MaMADS1*, *2*, *3* and *4* occurred later and in parallel with the increase in ethylene production in whole bananas. These results fit the suggestion that ripening starts in the pulp and then progresses to the peel (Dominguez and Vendrell, 1993). Moreover, our results support the idea that the initiation of climacteric respiration is not dependent on ethylene (Pech *et al.*, 2008). The decrease in firmness that was initiated before the ethylene peak (Fig. 3), supports the idea that the

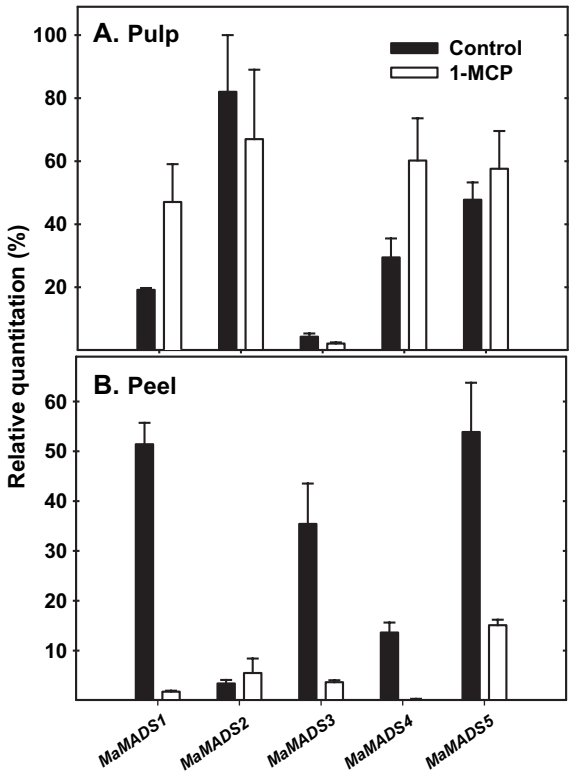

Fig. 7. Response to 1-MCP of *MaMADS*-box genes in peel and pulp. Banana fruits were treated with 0.3 μl l^{-1} 1-MCP at the onset of ethylene peak (8 d after harvest for 18 h). The expression was determined for control and 1-MCP-treated samples relative to other samples during fruit ripening and results of day 10 after harvest are presented.

initiation of some ripening processes start before the ethylene peak.

Interactions between ethylene and MADS-box gene expressions

In this study, exogenous ethylene and 1-MCP have been used to determine the involvement of ethylene in the *MaMADS* gene expression. Application of ethylene advanced the climacteric peak, and increased respiration, while 1-MCP inhibited ethylene production (data not shown), as has been reported previously (Golding *et al.*, 1998; Zhang *et al.*, 2006; Liu *et al.*, 2009). Using ethylene, it was possible to determine that *MaMADS3*, *4*, and *5* in the pulp, and *MaMADS1* and *3* in the peel are regulated by ethylene (Fig. 6). Since *MaMADS3* is induced by ethylene in the peel and pulp, it is suggested that its expression is controlled by an ethylene-induced transcription factor common to both peel and pulp. However, *MaMADS4* and *5* in the pulp and *MaMADS1* in the peel are most likely controlled by tissue-specific ethylene-induced transcription factors. These results may explain why, during the climacteric peak, *MaMADS4* and *5* are elevated mainly in the pulp and *MaMADS1* mainly in the peel in parallel to the climacteric peak. This further emphasizes the difference in ripening programmes which exist in the peel and in pulp.

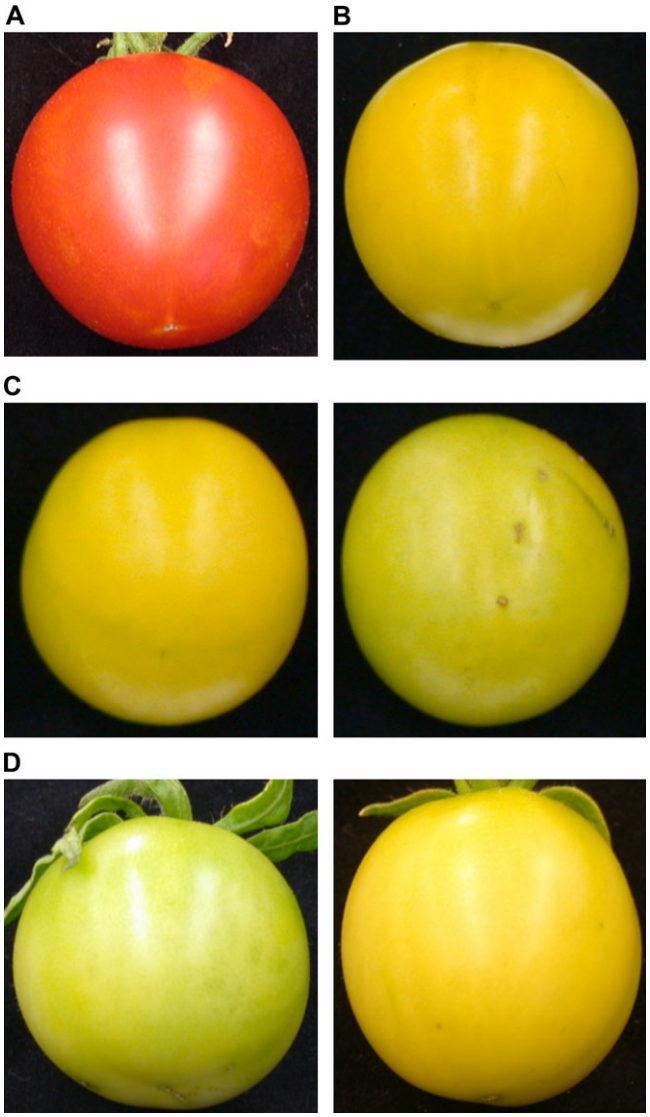

Fig. 8. Expression of *MaMADS* genes in *rin/rin* tomato fruit did not complement ripening. Ailsa Craig wild type, nearly isogenic *rin/rin*, and transgenic *rin/rin* tomato fruit were tagged at anthesis and designated as breaker stage at the same age as wild-type fruit which showed the first signs of colour change. Fruit were harvested 10 d post-breaker stage and photographed. Fruit are shown from (A) Ailsa Craig wt, (B) Ailsa Craig nearly isogenic for *rin/rin*, (C) *rin/rin* T_1 lines over-expressing *MaMADS1* (line 15 on the left and line 18 on the right), (D) *rin/rin* T_1 lines over-expressing *MaMADS2* (line 3 on the left and line 8 on the right).

The analysis of gene expression following 1-MCP applied at the onset of ethylene burst (Fig. 7), demonstrated that the expression of both *MaMADS4* and *MaMADS1* is negatively regulated by ethylene at the climacteric stage in pulp tissue. It is possible that early after harvest *MaMADS4* is induced by ethylene, but, during the climacteric peak, its expression is inhibited by ethylene. For *MaMADS1*, the expression increased by several-fold following the climacteric peak, suggesting that the inhibition observed during the climacteric peak is removed when ethylene is reduced.

On the other hand, in the peel, *MaMADS1*, *3*, and *5* are reduced by 1-MCP, further strengthening the conclusion that at least *MaMADS1* and *3* are ethylene-induced. Negative regulation of genes by ethylene at the climacteric peak has been demonstrated before in tomato (Hoeberichts *et al.*, 2002). Moreover, 1-MCP increased ethylene production in tomato when applied at the breaker stage and also in banana when applied at the yellow transition stage (Pelayo *et al.*, 2003). In another study, application of 1-MCP to propylene-treated fruit revealed that 1-MCP increased ethylene production concomitantly with *MaACS1* in the pulp and not in the peel (Inaba *et al.*, 2007). It is possible that, in the pulp, in comparison to peel, there is an additional control component of ethylene production which is responsible for a reduction in ethylene production and *MaMADS4* is involved in this control.

The unique expression of MaMADS2 *suggests that this gene plays an important role in banana fruit ripening*

Sequence similarity, in combination with similar temporal and spatial expression patterns, is necessary to establish orthologous relationships between MADS-box genes (Arora *et al.*, 2007). In the case of *MaMADS2* and *LeMADS-RIN*, it is clear that the two genes have very low similarity especially in their C-terminus, however, the expression pattern during ripening is very similar; both genes are induced at the onset of the climacteric (Fig. 4) (Vrebalov *et al.*, 2002).

Several studies on the involvement of MADS-box genes in flowering suggest that the functional similarity is not necessarily related to sequence similarity and the function of specific MADS-box genes can be taken by another type of gene. For instance, in rice, another monocot, *Arabidopsis*-related genetic switch systems control floral transition, but they are based on different MADS-box transcription factors (Andersen *et al.*, 2004). Similarly, a *SEP3* from lily, a monocot species, did not possess the predicted E class function during floral organ development, when expressed in *Arabidopsis* (Tzeng *et al.*, 2003). While neither *MaMADS2* nor *MaMADS1* was able to complement the tomato *rin* mutation, this result does not exclude a similar role for these genes in banana fruit ripening. Indeed, the ethylene-independent expression pattern of *MaMADS2* during banana fruit ripening suggests that this gene may regulate ripening and act upstream of the ethylene response pathway. Transgenic banana with reduced levels of MaMADS2 expression will be required to test this hypothesis.

In summary, our results suggest that several *MaMADS*-box genes may function during the climacteric and some may be induced by ethylene, but MaMADS2 is most likely serves as an upstream regulator, since it is not affected by ethylene. Our results also clearly support previous suggestions that ripening initiates in the pulp, possibly via an increase in CO_2 and ethylene produced by the pulp initiates ripening of the peel. It is also suggested that different components of the *MaMADS*-box genes participate in the ripening programs, all along the paper of these two tissues.

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