Banana *MaMADS* Transcription Factors Are Necessary for Fruit Ripening and Molecular Tools to Promote Shelf-Life and Food Security[1][OPEN]

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Genetic solutions to postharvest crop loss can reduce cost and energy inputs while increasing food security, especially for banana (*Musa acuminata*), which is a significant component of worldwide food commerce. We have functionally characterized two banana E class (*SEPALLATA3* [SEP3]) MADS box genes, *MaMADS1* and *MaMADS2*, homologous to the tomato (*Solanum lycopersicum*) *RIN-MADS* ripening gene. Transgenic banana plants repressing either gene (via antisense or RNA interference [RNAi]) were created and exhibited specific ripening delay and extended shelf-life phenotypes, including delayed color development and softening. The delay in fruit ripening is associated with a delay in climacteric respiration and reduced synthesis of the ripening hormone ethylene; in the most severe repressed lines, no ethylene was produced and ripening was most delayed. Unlike tomato *rin* mutants, banana fruits of all transgenic repression lines responded to exogenous ethylene by ripening normally, likely due to incomplete transgene repression and/or compensation by other MADS box genes. Our results show that, although MADS box ripening gene necessity is conserved across diverse taxa (monocots to dicots), unlike tomato, banana ripening requires at least two necessary members of the *SEPALLATA* MADS box gene group, and either can serve as a target for ripening control. The utility of such genes as tools for ripening control is especially relevant in important parthenocarpic crops such as the vegetatively propagated and widely consumed Cavendish banana, where breeding options for trait improvement are severely limited.

Fruits of plants in the genus *Musa*, including bananas and plantains, represent staple foods for millions of people, especially in developing countries, and are an important carbohydrate and nutrient source for billions more the world over. In developing (including many producing) countries, postharvest methodologies to extend shelf-life are minimal and losses are correspondingly high. Regulating ripening and extending banana shelf-life via genetic means will facilitate developing country food security in addition to saving money and energy in countries where high-input postharvest practices predominate.

Genetic and postharvest methods for extending fruit shelf-life have been achieved in the model plant tomato (*Solanum lycopersicum*), and genetic ripening mechanisms have been thoroughly studied in this fleshy fruit, providing numerous candidate gene targets for other important fruit crops, including banana (*Musa acuminata*; Klee and Giovannoni, 2011). Banana and tomato fruit exhibit climacteric (elevated) respiration and a surge in ethylene hormone production concomitant with ripening (Seymour, 1993; Klee and Giovannoni, 2011), and tomato genes necessary for ethylene production and climacteric respiration have been identified (Alexander and Grierson, 2002; Giovannoni, 2007; Pirrello et al., 2009). Among them are several MADS box genes: *SlMADS-RIN* of the *SEPALLATA* (*SEP*) *E* function clade (Vrebalov et al., 2002; Ito et al., 2008; Zhong et al., 2013); *FRUITFULL* (*FUL1* and *FUL2*) homologs (Bemer et al., 2012; Shima et al., 2014); and *TAGL1*, an *AGAMOUS* (*AG*)-like MADS box gene necessary for both early fruit expansion and later ripening (Itkin et al., 2009; Vrebalov et al., 2009). The SlMADS-RIN

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protein interacts with additional MADS box proteins, including TAGL1 and SlTAGL11 (Leseberg et al., 2008) and the tomato FUL homologs FUL1 and FUL2, to execute ripening (Martel et al., 2011; Fujisawa et al., 2014). In one case, a member of the *SEP* clade, *SlMADS1*, has been suggested to act as a negative regulator (Dong et al., 2013). Reduction in the expression of any of these ripening-associated MADS box family members (except *SlMADS1*), whether by transgenic manipulation or naturally by mutation, measurably delayed fruit ripening progression (Klee and Giovannoni, 2011; Seymour et al., 2013). For example, either down-regulation of *SlMADS-RIN* or a spontaneous mutation, resulting in its C-terminal deletion, caused an inhibition of climacteric respiration and of ethylene production, even in heterozygote mutants, indicating that this gene acts in a dose-dependent manner upstream of ethylene production (Kitagawa et al., 2005). Tomato *SlMADS-RIN* exerts an ethylene-independent effect on ripening downstream of ethylene action, since exogenous application of ethylene to *rin/rin* tomato fruit does not restore ripening (Lincoln and Fischer, 1988; Giovannoni, 2001; Vrebalov et al., 2002). The naturally occurring loss-of-function mutation in the *SlMADS-RIN* gene is widely used in commercial hybrid tomato germplasm the world over for ripening and shelf-life control. The practical success of this tomato mutation validates the utility of targeting RIN-MADS ortholog function in other species to effectively control ripening in a commercially viable manner.

Similar MADS box ripening genes have been described in other fruits (Rigola et al., 1998; Yao et al., 1999; Sung et al., 2001; Boss et al., 2002), including strawberry (*Fragaria* spp.), a nonclimacteric fruit that ripens in the absence of elevated respiration or ethylene (Seymour et al., 2011). In apple (*Malus domestica*), suppression of a *SEP* gene (*MdMADS8/9*) reduced the expression of ripening-related genes and resulted in altered fruit morphology (Ireland et al., 2013; Schaffer et al., 2013). Somewhat surprisingly, ripening MADS box genes in most other important fruit crops remain poorly investigated despite the enormous potential toward improving fruit storage, transport, and associated impacts on food security. This may in part reflect the difficulty of transforming many fruit crops. Toward this end, we focused on banana, a crop of worldwide importance, a major food commodity, and a staple crop in areas of Africa and Asia. Banana additionally represents the monocot lineage of plants, which is not well characterized at the molecular level in terms of fleshy fruit ripening.

We previously described the temporal expression during ripening of six banana fruit-associated *MaMADS* box genes in peel and pulp. *MaMADS1* to *MaMADS4* may function similarly to RIN in tomato, being *SEP* genes (Elitzur et al., 2010), and *MaMADS5* may function like *TAGL1*, being an AG gene (Itkin et al., 2009; Vrebalov et al., 2009). MaMADS5 was found to bind to CArG box sequences, which exist in the promoters of major ripening genes (Choudhury et al., 2012) and recently was found to bind to OVATE FAMILY PROTEIN (Liu et al., 2015b), a suppressors of cell elongation (Wang et al., 2007). The expression of the *MaMADS1* to *MaMADS5* genes was induced during ripening with gene-specific variation in the pulp and peel, with *MaMADS1* most highly ripening induced. Some of these genes were ethylene inducible in peel and/or pulp, but *MaMADS2* was not induced by ethylene in either (Elitzur et al., 2010; http://banana-genome.cirad.fr/cgi-bin/gbrowse/musa_acuminata/?name=GSMUA_Achr1P12150_001). Together, these results suggest *MaMADS1* and *MaMADS2* as logical candidates for important ripening functions, representing the most highly ripening-expressed gene and operating upstream of ethylene, respectively. To functionally define the roles of *MaMADS1* and *MaMADS2* in banana fruit development, we generated transgenic banana plants repressing each gene individually and show that each one necessary for ripening. These results provide evidence that MADS box ripening function is an ancient activity of this gene family (as it is common to both monocots and dicots) and further demonstrate their clear practical utility as genetic targets for controlling shelf-life and quality in a vegetatively propagated (and thus less amenable to breeding solutions) fruit crop that is utilized the world over and important to food security and international commerce.

RESULTS

Transgenic Banana Plants with Reduced Expression of *MaMADS1* or *MaMADS2*

Banana fruit start to produce ethylene in the pulp and later in the peel (Supplemental Fig. S1), indicating that ripening is initiated in the pulp. We previously described the expression of six *MaMADS* box genes from banana fruit (Elitzur et al., 2010), and based on RNA sequencing (RNAseq) data, we discovered seven additional highly expressed *MaMADS* box genes (more than 50 reads per kilobase of transcript per million reads mapped [RPKM]) in the pulp of banana. Homologs of *SlFUL1/2*, *SlTAGL1*, and *SlMADS-RIN* were identified and used to construct a phylogenetic tree (Supplemental Fig. S2). *MaMADS2* and *MaMADS1* are most similar to SlMADS-RIN, with 62% and 56% amino acid identity, respectively (Elitzur et al., 2010).

We proceeded to functionally characterize the *MaMADS1* and *MaMADS2* genes, whose expression is highly elevated in the pulp in concert with ripening. Furthermore, *MaMADS2* expression was not affected by ethylene in the pulp, suggesting that at least *MaMADS2* expression may precede (and thus mediate) the induction of key ripening processes. These *MaMADS* box genes contain four domains: M, I, K, and C, with the M domain most conserved. The C region is the most divergent, as is typical of MADS box genes (Elitzur et al., 2010); therefore, all three repression constructs targeted the C region (Supplemental Fig. S3). Specifically, three repression constructs were created: (1) the 195-bp RNAi *MaMADS1* targets the K and C domains; (2) RNAi *MaMADS2* targets

the C domain and the 3′ untranslated region (UTR; 302 bp); and (3) antisense (AS) *MaMADS2* targets the same sequence as for (2). The construct used for *MaMADS1* silencing has 61% homology with *MaMADS2*, and the longest stretch of identity is 11 nucleotides. The construct used for *MaMADS2* silencing has only 38% homology with *MaMADS1*, and the longest stretch of identity is seven nucleotides. Identities of up to 51% with stretches of up to 12 nucleotides resulted in gene-specific silencing in a gene family in rice (*Oryza sativa*; Miki et al., 2005). The above DNA targets are depicted in Supplemental Figure S3 and detailed in "Materials and Methods." The constructs were used for the transformation of banana embryo cultures, and transgenic plants were recovered as described in Table I. Twenty-six plants were recovered from RNAi *MaMADS1* transformation, 19 from RNAi *MaMADS2*, and 18 from two independent transformations of AS *MaMADS2*. The transformation of all three constructs yielded fertile plants verified for inserts using leaf DNA as described in "Materials and Methods" with no obvious growth phenotype.

Banana fruit were harvested from independent transgene-confirmed plants of all three transgenic classes (RNAi *MaMADS1*, RNAi *MaMADS2*, and AS *MaMADS2*), in addition to wild-type untransformed control plants propagated at the same time. Target gene mRNA levels at the breaker (color transition) stage in peel and pulp were determined by quantitative real-time (Q-RT)-PCR (Fig. 1). *MaMADS1* or *MaMADS2* mRNA accumulation in control plants was high in peel and pulp, as expected from prior results (Elitzur et al., 2010). *MaMADS1* mRNA was low in peel and pulp of RNAi *MaMADS1* lines, and *MaMADS2* mRNA was low in both RNAi *MaMADS2* and AS *MaMADS2* peel and pulp for all transgenic plants examined. The specificity of the respective silencing constructs was examined by determining *MaMADS1* mRNA expression in *MaMADS2*-suppressed fruit and *MaMADS2* mRNA in *MaMADS1*-suppressed fruit. Down-regulation of *MaMADS1* did not substantially reduce the levels of *MaMADS2* in the pulp but did reduce it in the peel, suggesting a regulatory rather than transgene homology effect in the peel. Down-regulation of *MaMADS2* substantially induced *MaMADS1* in the pulp at the breaker stage but reduced it in the peel in both RNAi *MaMADS2* and AS *MaMADS2* fruit. The reduction of the target genes in both peel and pulp, yet tissue-specific repression of the alternate gene (and induction of *MaMADS1* in *MaMADS2* repression lines), are consistent with a regulatory repression rather than a transgene homology effect.

MaMADS1 and MaMADS2 Are Both Necessary for Ripening

In order to ascertain the impact of MaMADS1 or MaMADS2 repression on ripening, we monitored transgenic and control fruit prior to and through ripening during postharvest storage at room temperature. Wild-type banana fruit ripened 12 to 13 d after harvest, while *MaMADS1*- and *MaMADS2*-suppressed fruits were delayed in the ripening transition by 3 to 14 d (Supplemental Table S1). Transgenic and control fruit (fingers) and hands (banana finger clusters) are shown in Figure 2, where at 20 d postharvest the control fruit show severe decay, while transgenic fruit remain yellow or green with little damage even at 26 d (Fig. 2). Fruit color, firmness, and total soluble solids (TSS) also were measured in the fruit of wild-type and transgenic plants (Fig. 3; Supplemental Table S1). In general, in all fruits of transgenic plants, breaker occurred later than in controls. More specifically, changes in color in the control plants occurred following the ethylene production burst (Figs. 3 and 4), while in transgenic plants, especially those that did not produce ethylene, it occurred either following or concomitant with the increase in respiration. At this stage, fruits of transgenic plants had higher firmness than wild-type fruit. These results demonstrate a strong influence on both time to onset of ripening and shelf-life of the *MaMADS1* and *MaMADS2* transcription factors. TSS of the peel and pulp were, in most lines, similar to those in the wild type yet correlated with the delayed-ripening transgene effects. These results further demonstrate the utility of using these genes for extending shelf-life across all important quality phenotypes, similar to the effects of the widely used *rin* mutation for tomato shelf-life extension.

MaMADS1 and MaMADS2 Affect Ethylene and Respiration

To further define the effects of these banana MADS box genes on ripening, respiration and ethylene production were measured on control and transgenic fruits

Table I. *Description of banana transgenic lines*

All lines were transferred to the field in April 2008. Letters indicate the molecule sections used for transformation as described in Supplemental Figure S3: C, full length of the C region; pK, partial length of the K region; 3′UTR, UTR at the 3′ end.

Description	Transfer to Greenhouse	Harvest	PCR-Positive Plants	Plant Marks
MaMADS1 pK + pC RNAi	October 16, 2007	May 5, 2009	29[a]	4-19, 4-20 (2-8, 2-18, 2-21)[b]
MaMADS2 C + 3′UTR RNAi	September 6, 2007	June 2, 2009	19	3-21, 3-23, 3-24, 2-32
MaMADS2 C + 3′UTR AS[c]	September 6, 2007	June 2, 2009	2	3-45, 2-44
	September 6, 2007	June 2, 2009	16	3-36, 3-37, 3-40

[a]Three plants died following their transfer to the field. [b]In parentheses are plants that exhibit chimeras as described in Figure 5. [c]Two independent transformations were performed for this construct.

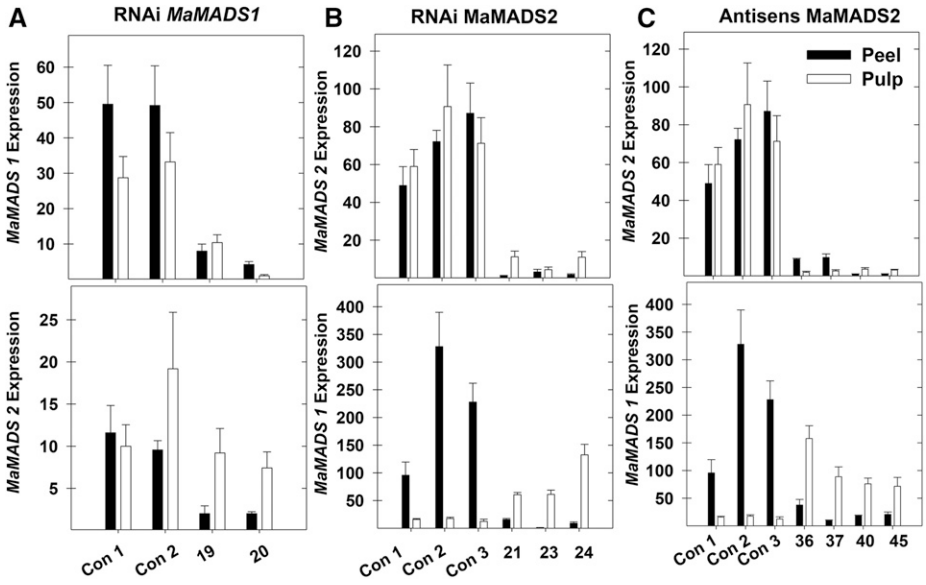

Figure 1. Determination of *MaMADS1* and *MaMADS2* expression levels in the repressed lines. *MaMADS1* and *MaMADS2* expression was determined in the targeted lines (top) and in the reciprocal repressed lines (bottom). The expression was determined in two independent lines of RNAi *MaMADS1* transgenic plants (A), in three lines of RNAi *MaMADS2* (B), and in four lines of AS *MaMADS2* (C). Expression was measured in peel and pulp of fruit at breaker stage (color change). Sampling times were as follows: for control fruit in A, 12 d after harvest (DAH; Con 1) and 14 DAH (Con 2); for control fruit in B and C, 10 DAH; for *MaMADS1* RNAi in A, 19 DAH (plant 19) and 23 DAH (plant 20); for RNAi *MaMADS2* in B, 16 DAH (plants 21, 23, and 24); and for AS *MaMADS2* in C, 19 DAH (plant 36), 16 d (plant 37), and 24 DAH (plants 40 and 45). For each analysis, RNA was extracted twice from two bananas, and preparations were examined two times each for technical replicates.

at consecutive days from harvest until the appearance of brown spots (Fig. 4). In each of the control I groups, typical climacteric respiration occurred at 9 to 12 days after harvest (DAH), preceding the expected burst in ethylene production (12–15 DAH; Fig. 4A). Fruit of two independently transformed RNAi *MaMADS1* plants were analyzed, and in both, respiration and ethylene were delayed or attenuated (Fig. 4B). These observations confirm the necessity of MaMADS1 for ripening via the control of climacteric respiration and ethylene hormone synthesis.

The development of plant chimeras with the transgene further corroborated the involvement of *MaMADS1* in fruit ripening. Besides the banana bunches harvested from the lines mentioned above, a number of bunches from other lines were not uniform in their color development after harvest, and bananas derived from the fifth hand exhibited earlier ripening than bananas from upper (older) hands (Fig. 5A). To further elucidate this phenomenon, the DNA of banana from different hands of the same bunch of three different lines was examined (Fig. 5B). It can be seen that, in three plants that harbor the correct insert (as judged by comparison with leaf PCR), no inserts was observed in the fifth hand, and only in two lines was an insert observed in the second hand (the control gene was amplified in all samples; data not shown), suggesting that the transformation yielded some chimeric plants. These results were in agreement with ethylene and carbon dioxide production for fruits of different hands (Fig. 5C). In control fruits, the ethylene production peak occurred on day 12 after harvest, even later than in bananas that did not harbor the insert (11 d in the second hand of line 21 and 7 or 9 d in the fifth hand of lines 21 and 18 or 8, respectively). However, the ethylene and carbon dioxide peaks appeared on day 16 after harvest in fruits that harbor the insert (second hand of lines 8 and 18).

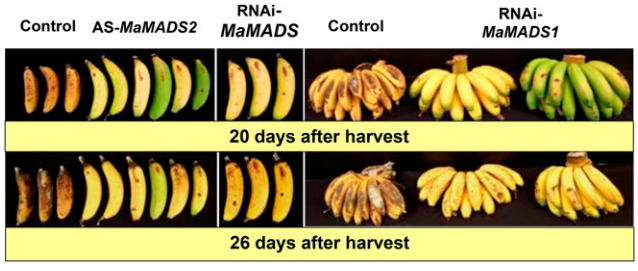

Figure 2. Banana fruits of control and *MaMADS1*- or *MaMADS2*-repressed transgenic plants. Individual banana fruit are presented from each of the transgenic AS *MaMADS2*, RNAi *MaMADS2*, and control plants and from whole hands of RNAi *MaMADS1* and control plants. Fruits of the first and second hands were placed at 20°C and photographed at 20 and 26 DAH.

Fruits from RNAi *MaMADS2* transgenic plants also showed reduced respiration and ethylene production (Fig. 4, C and D), while even greater inhibition of ethylene production and respiration was observed in banana fruit from AS *MaMADS2* transgenic plants (Fig. 4E). In two AS *MaMADS2* plants (AS 40 and AS 45), the fruit did not show any increase in ethylene, and in these fruits, respiration was attenuated and increased gradually starting

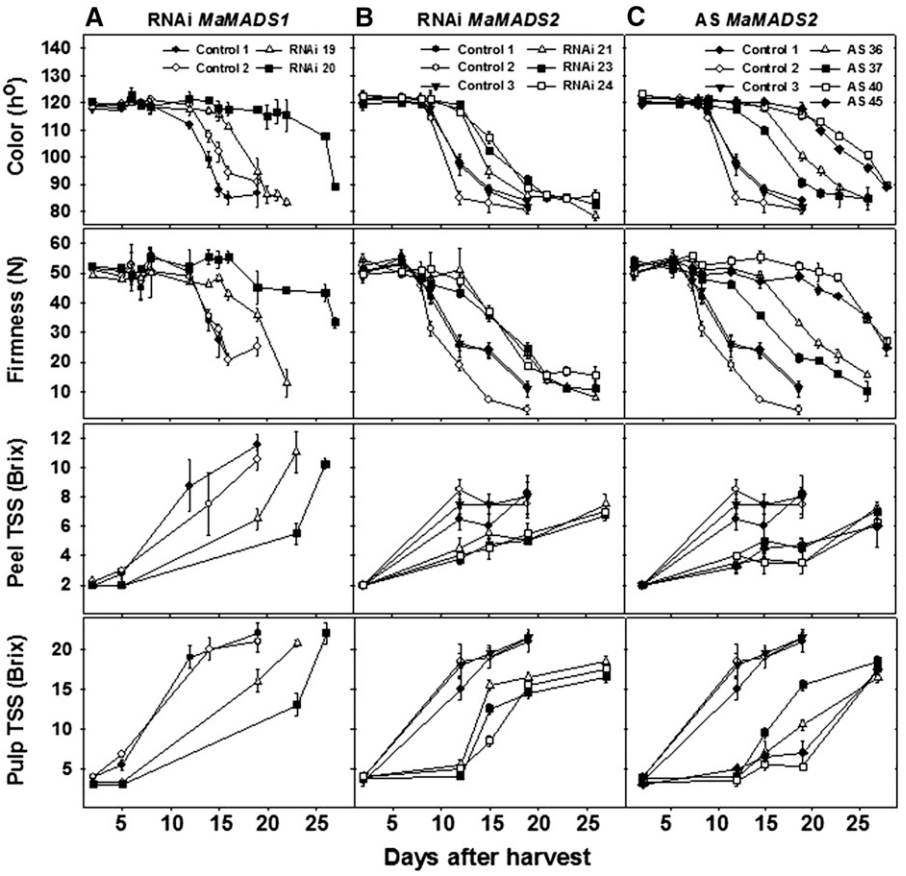

Figure 3. Quality parameters of *MaMADS1*- and *MaMADS2*-repressed banana fruit. The parameters of color (h°), firmness (N), and TSS (Brix) in peel and pulp were examined after harvest in RNAi *MaMADS1* (A), RNAi *MaMADS2* (B) and AS *MaMADS2* (C) and in the corresponding control plants. Each time point is an average of at least six fruits ± SE. A summary of the parameters is described in Supplemental Table S1.

26 DAH. In two additional plants, the ethylene production peak was lower than that of controls and occurred later than in the control (10 DAH, control; 18 DAH, AS 36; and 15 DAH, AS 37). These ethylene peaks occurred in parallel with increased respiration.

Repression of *MaMADS1* or *MaMADS2* Modified the Expression of Other MaMADS Box Genes

To further assess the effects of transgene suppression, we determined the levels of expression in *MaMADS1*- and *MaMADS2*-repressed lines of *MaMADS3* to *MaMADS5* (Fig. 6). *MaMADS3*, a *SEP* gene with lower identity to *MaMADS1/2* (52% and 54% identity to MaMADS1 and MaMADS2, respectively), showed reduced expression in both types of transgenic plants in comparison with wild-type fruit at breaker in peel. The possibility of *MaMADS3* targeting, by either transgene or both transgenes, remains a formal possibility, although the lower level of homology of this gene to *MaMADS1* or *MaMADS2* sequences used for silencing (39% and 28.5%, respectively) would argue against direct transgene effects and suggests that *MaMADS3* is under regulatory constraints imposed by both genes. On the other hand, the levels of both *MaMADS4* (a *SEP* gene) and *MaMADS5* (an *AG* gene) increased in the pulp of *MaMADS2*-repressed plants. The levels of *MaMADS5* also increased in the pulp of *MaMADS1*-repressed plants (Fig. 6). These results suggest that *MaMADS4* and *MaMADS5* genes are under negative control of *MaMADS2* and *MaMADS5*, possibly also under the negative control of *MaMADS1* in the pulp. Alternatively, these results represent a compensatory mechanism. They are consistent with regulatory rather than direct transgene effects. The up- and down-regulation of additional MADS box genes is consistent with reports in tomato, where a number of MADS box genes are repressed in the *rin* mutant (Martel et al., 2011; Zhong et al., 2013) and some are repressed and others are induced in transgenic *TAGL1* repression lines (Itkin et al., 2009; Vrebalov et al., 2009). These altered expression patterns in both banana and tomato represent downstream regulatory effects or compensatory mechanisms. While the specific functions of some of these responses to reduced-ripening MADS box gene expression remain to be determined, in no instance do they detract from the mutant- or transgene-demonstrated necessity of genes such as *MaMADS1* or *MaMADS2* for normal ripening manifestation.

MaMADS1- and *MaMADS2*-Repressed Banana Fruit Retain Ethylene Sensitivity

It was clear that normal ripening was delayed in fruit of the transgenic plants, and it was important to determine how these fruit respond to ethylene treatment, both to clarify their ripening responses and to enable their practical utilization, as ripening-delayed fruit require

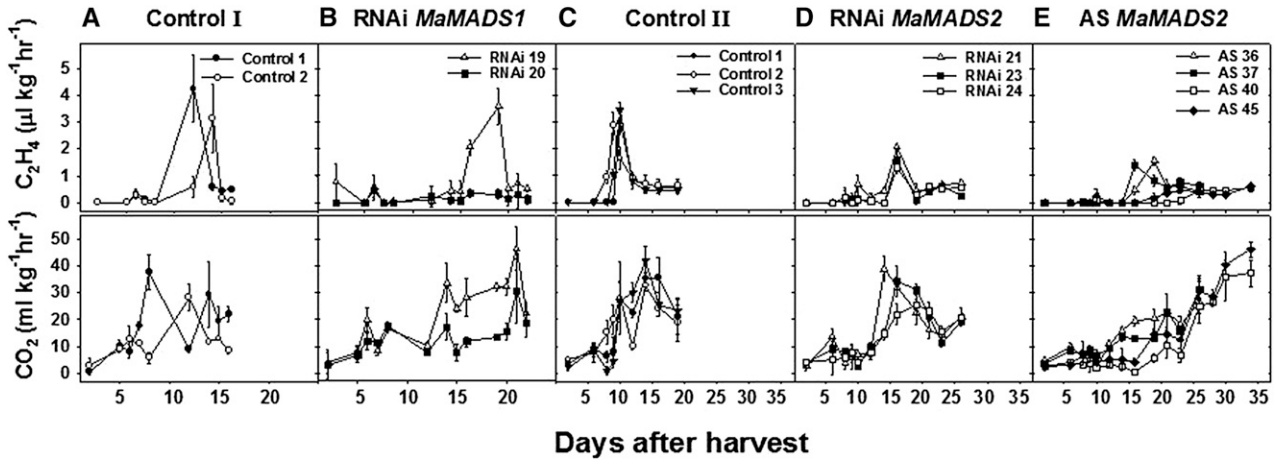

Figure 4. Comparison of ethylene (C_2H_4) and carbon dioxide (CO_2) production after harvest between control and repressed *MaMADS1* and *MaMADS2* fruits. Production was determined for control fruits (A and C) in two independent experiments: control I (A) and control II (C). Control I fruit served as a control for transgenic RNAi *MaMADS1* (B) and control II for either RNAi *MaMADS2* (D) or AS *MaMADS2* (E). Each time point is an average of at least six fruits ± SE.

ethylene responsiveness. Banana fruit of the control and transgenic plants were exposed to ethylene. Fruit of the *MaMADS*-repressed lines responded to ethylene in a similar manner to control fruit and developed yellow color (Fig. 7A). In addition, treatment with ethylene increased respiration and ethylene production (Fig. 7B), at least in one AS *MaMADS2* line (line 37). Further examination of both the firmness and color in fruits from several repressed lines showed that there were no differences between the wild type and any of the transgenic plants following ethylene treatment (Supplemental Fig. S4). These results demonstrate that, while MaMADS1 and MaMADS2 are necessary for endogenous ethylene production and ripening, ripening-delayed fruit repressed in

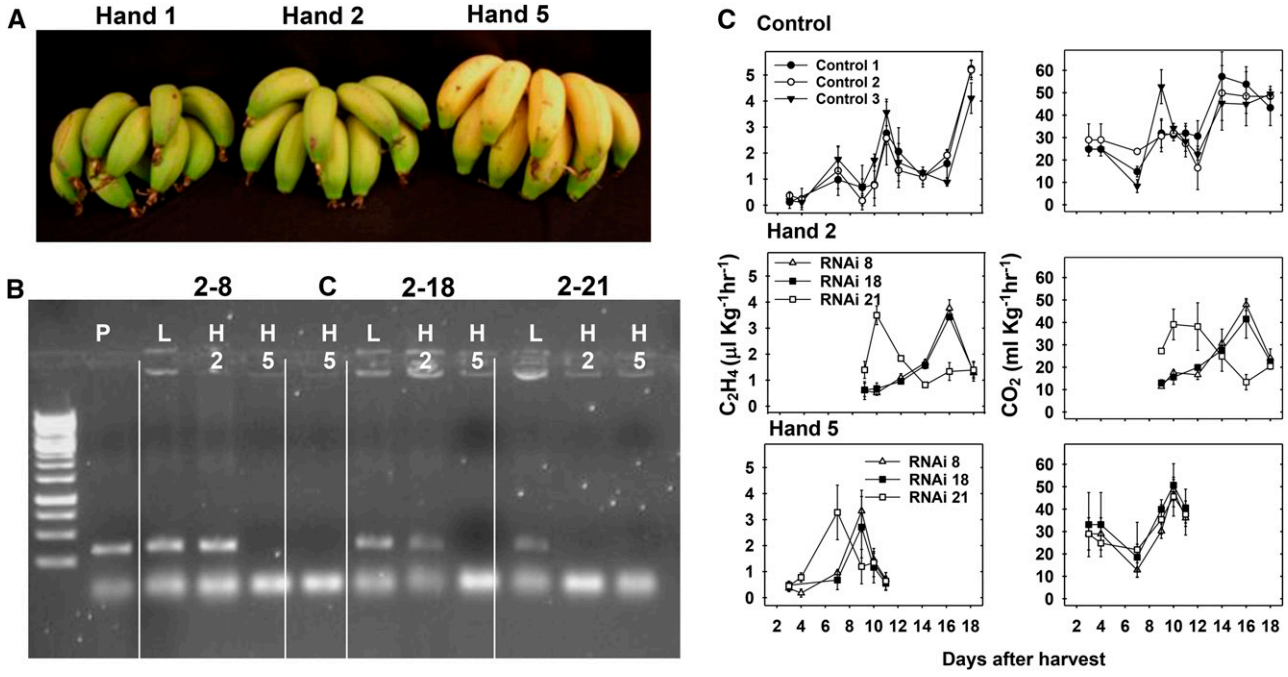

Figure 5. Description of chimeras obtained from transgenic plants harboring the RNAi *MaMADS1* constructs. A, Photograph of fruit from hands 1, 2, and 5 of plant 2-18. B, Confirmation of the insert in DNA extracted from leaves of three independent banana plants (2-8, 2-18, and 2-21) in comparison with control plants. The primers used for confirmation are described in Supplemental Table S3 (reaction a). C, Nontransgenic control plant; H, hand number; L, leaf; P, positive plasmid. C, Ethylene (C_2H_4; left) and carbon dioxide (CO_2; right) production in control plants and in hands 2 and 5 taken from transgenic plants. Each time point is an average of at least six fruits ± SE.

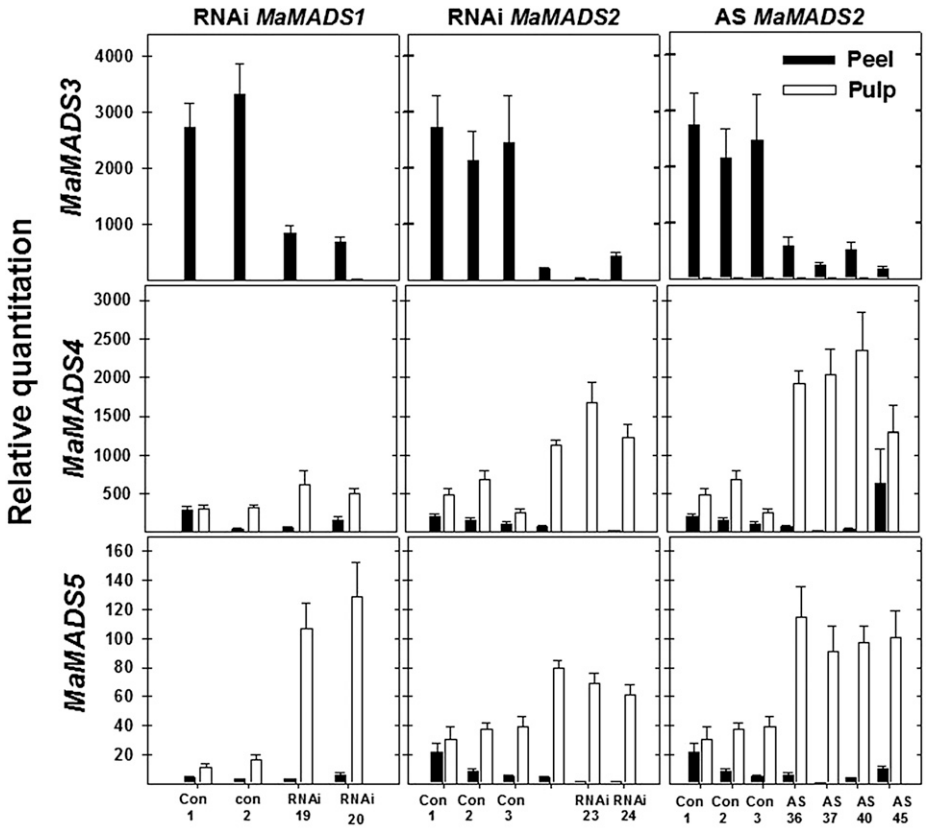

Figure 6. Expression patterns of *MaMADS3*, *MaMADS4*, and *MaMADS5* in *MaMADS1*- and *MaMADS2*-suppressed fruits at breaker. Expression was determined in *MaMADS1* RNAi and *MaMADS2* RNAi/AS transgenic plants. Sampling times were as follows: control for RNAi *MaMADS1* fruit, 12 DAH for Con 1 and 14 DAH for Con 2; for the suppressed lines, 19 DAH (plant 19) and 23 DAH (plant 20); control for RNAi/AS *MaMADS2*, 10 DAH; 16 DAH for plants 21, 23, and 24 (B); 19 DAH for plant 36; 16 DAH for plant 37; and 24 DAH for plants 40 and 45. For each analysis RNA was extracted twice from two bananas, and preparations were examined two times each for technical replicates.

either gene are still responsive to ethylene treatment necessary for optimal market utility and performance.

Ethylene Alters the Expression of Multiple MaMADS Box Genes in *MaMADS2*-Repressed Fruit

To further clarify if the *MaMADS2*-repressed lines respond to ethylene by modifying fruit *MaMADS* box gene expression patterns, we analyzed multiple fruit *MaMADS* box genes that are homologs to genes known to affect ripening in tomato. Banana fruits of AS *MaMADS2* (line 37) and the control were treated at harvest with ethylene, tissue was collected immediately following ethylene treatment, and the levels of fruit MaMADS box genes' expression were compared between ethylene-treated and nontreated wild-type and AS *MaMADS2* (line 37) plants. The expression of *MaMADS1*, *MaMADS4*, and *MaMADS9* was induced by ethylene in both the control and the repressed line, and *MaMADS5* was repressed by ethylene in the *MaMADS2* line, although to levels still approximately 50% higher than in controls with or without ethylene. *MaMADS7*, *MaMADS8*, *MaMADS10*, and *MaMADS11* showed little change in response to either *MaMADS2* reduction or ethylene. Whether ethylene confers ripening in *MaMADS2*-repressed lines in the context of residual *MaMADS2* or via other functionally redundant MaMADS gene(s) remains to be determined, as does the relevance of the altered expression of additional MaMADS box genes due to reduced *MaMADS2* expression or ethylene. The induction of *MaMADS1* by ethylene remains consistent with the possibility that MaMADS1 and MaMADS2 are functionally equivalent, although the clear ripening-delay phenotypes resulting from either *MaMADS1* or *MaMADS2* repression confirm that wild-type levels of both genes are necessary for ripening, absent any external influence such as the application of exogenous ethylene. Testing the potential functional redundancy would require specific induction of *MaMADS1* (presumably via a transgene) in *MaMADS2*-repressed lines.

DISCUSSION

Banana is one of the most important fruit crops the world over, consumed in most countries and affecting food security, agriculture, and economics, especially in developing countries. Maintaining quality and shelf-life is a major source of product cost in developed countries and the basis of loss, reduced value, and diminished food security in developing countries. The Cavendish desert banana is an important cash crop and trade commodity, with over 16 million metric tons exported in 2012 (http://www.fao.org/docrep/019/i3627e/i3627e.pdf). Delayed ripening and extended postharvest shelf-life with minimal inputs have great potential to increase banana value, increase effective yield, and promote food security, absent additional environmental pressure (i.e. the need to increase production area). As the Cavendish banana is parthenocarpic and vegetatively propagated, there are

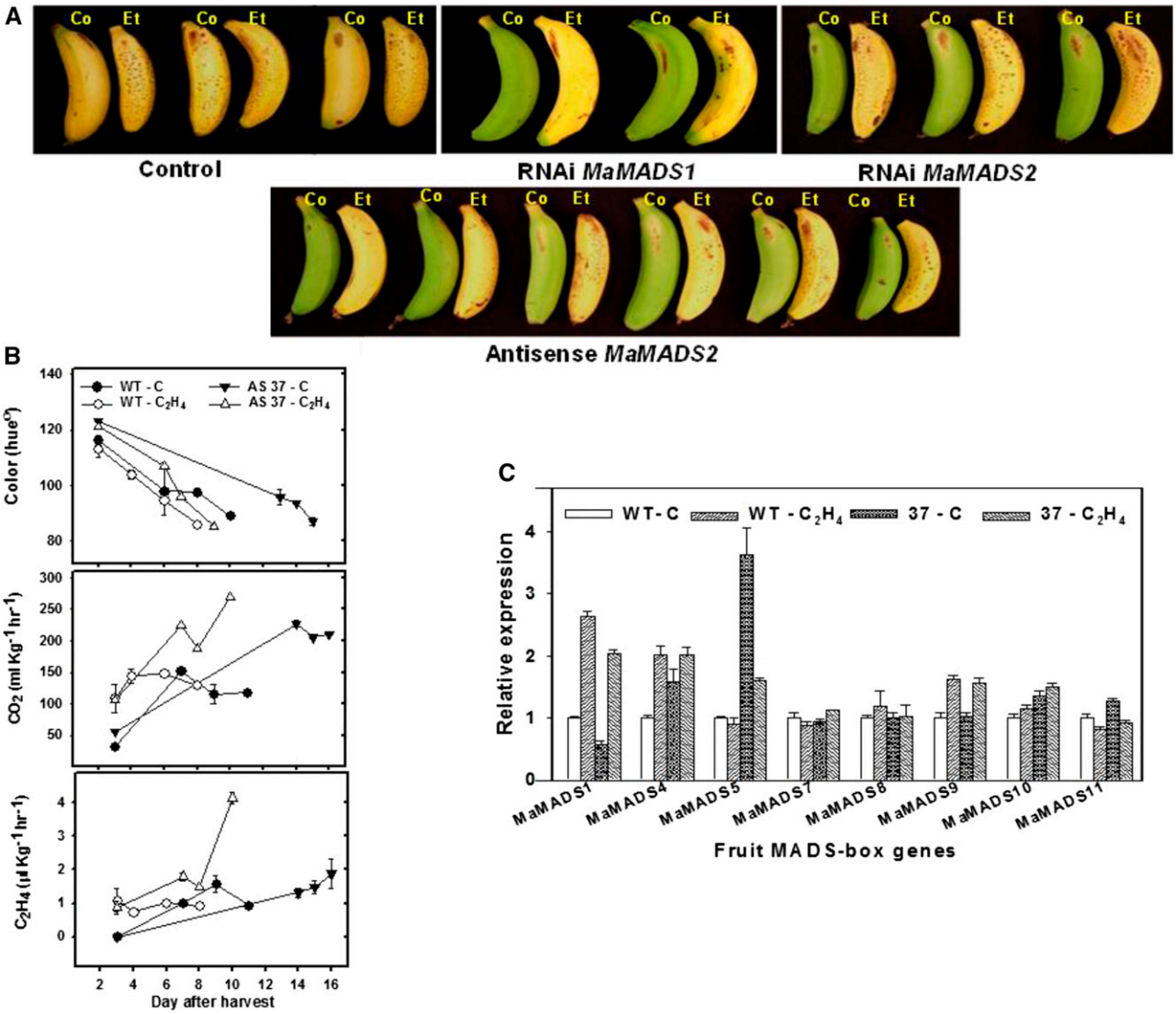

Figure 7. Response to ethylene of banana fruits from control and *MaMADS1*/*MaMADS2*-suppressed transgenic plants. A, Fruits of ethylene-treated (Et) and nontreated (Co) control, RNAi *MaMADS1*, RNAi *MaMADS2*, and AS *MaMADS2* plants are displayed. B, Fruits were treated with ethylene (10 μL L^{-1} for RNAi *MaMADS1* or 1 μL L^{-1} for control and *MaMADS2*-suppressed lines) immediately after harvest for 1 d and photographed following 12 d. Color, carbon dioxide (CO_2), and ethylene evolution (C_2H_4) were determined following ethylene treatment of AS *MaMADS2* (AS 37). Each time point is an average of at least six fruits ± SE. Additional quality parameters are displayed in Supplemental Figure S4. C, Expression analysis of ripening-related MaMADS box banana fruit in the wild type (WT) and AS *MaMADS2* (line 37) following ethylene treatment. Fruits for B and C were treated 1 d after harvest with 10 μL L^{-1} ethylene for 1 d. Immediately following treatment, tissues were collected and gene expression was determined by Q-RT-PCR. For each analysis, RNA was extracted twice from two bananas, and preparations were examined two times each for technical replicates. C, Nontreated samples; C_2H_4, ethylene-treated samples.

limited options for genetic improvement. Identifying key ripening genes to promote genetic modification via gene editing, transgene integration, or breeding (when possible) is a key route to banana improvement.

MaMADS1 and MaMADS2 Are Necessary for Banana Ripening

Much of our knowledge of fleshy fruit ripening is derived from the model dicot tomato, and it is not clear whether all fruit, including those of monocots like banana, employ similar genetic mechanisms. Tomato, however, does provide numerous candidate genes for the identification and testing of functional homologs in other important fruit species. One such well-studied tomato gene is *SlMADS-RIN*, whose mutation is widely used for tomato ripening control and shelf-life extension (Vrebalov et al., 2002), validating the practical value of regulating this ripening factor in a commercially viable manner. Here, we show, through transgene-mediated

silencing, that the MaMADS box genes *MaMADS1* and *MaMADS2* are necessary genetic components of ripening. Furthermore, as ethylene is induced in pulp prior to peel, we suggest that changes occurring in pulp are central to initiating fruit ripening and result in a cascade of hormone and additional transcription factor activities that mediate the full process in both peel and pulp. As an example, *MaMADS3*, which shows very low homology to either *MaMADS1* or *MaMADS2*, is repressed in peel of both *MaMADS1*- and *MaMADS2*-repressed lines due to the reduction in ethylene occurring in these fruit. Its expression is recovered with ethylene, as shown previously (Elitzur et al., 2010).

The specificity of transgene silencing is supported by the facts that the expression of other *MaMADs* box genes either did not change or was altered in a tissue-specific manner, inconsistent with targeted transgene repression (Figs. 1 and 6) and more likely reflecting the regulatory interactions among ripening MADS box genes also reported in tomato (Zhong et al., 2013). How these additional regulators participate in the ripening cascade requires further investigation. In contrast to the single tomato *SEP* regulator *SlMADS-RIN*, in banana, both E class (*SEP*) *MaMADS1* and *MaMADS2* genes are necessary for ripening. The central ripening function of the *SEP* MADS box genes is clearly an ancient activity of this gene family, as it is common to both monocots and dicots, although evolution has tailored common regulators to distinct fruit outcomes.

Reducing the transcript levels of either *MaMADS1* or *MaMADS2* (Fig. 1) decreased ripening progression, as was demonstrated by the inhibition in color change, delayed softening, and slower accumulation of sugar in the pulp and peel (Figs. 2 and 3). The fruits of transgenic banana lines, once ripened, had similar characteristics to control fruits, which matured many days earlier (Fig. 3; Supplemental Fig. S1). This phenomenon likely reflects the activity of residual target gene activity in all transgenic repression lines and is supported by a similar dose response in tomato lines heterozygous for the *rin* mutation. Silencing either gene delayed and reduced ethylene production or completely abolished it. However, climacteric respiration (as determined by carbon dioxide evolution), although delayed in *MaMADS1*- and *MaMADS2*-suppressed fruits, eventually reached wild-type fruit rates, even in lines that never demonstrated elevated ethylene synthesis (Figs. 3 and 4). The phenotypes of the described transgenic banana plants indicate that both the *MaMADS1* and *MaMADS2* genes are high-value targets for banana ripening and shelf-life improvement.

MaMADS2 Acts Upstream in the Ripening Cascade

MaMADS2 expression increases at the onset of ripening yet was not modified in peel and pulp by ethylene, indicating that it may act upstream of this hormone's ripening-related synthesis (Elitzur et al., 2010). The fact that ethylene synthesis and ripening is delayed upon *MaMADS2* repression is consistent with this hypothesis. Moreover, *MaMADS2* negatively regulates the expression of *MaMADS4* and *MaMADS5* in pulp at breaker and at harvest (Figs. 6 and 7C) and, to a lesser degree, *MaMADS10* and *MaMADS11* (homologs of *FUL1* and *FUL2*) at harvest (Fig. 7C) and *MaMADS1* at breaker (Fig. 6), at the time of its induction (Elitzur et al., 2010). *MaMADS2* expression in pulp was not affected in *MaMADS1*-repressed fruit (Fig. 1), confirming a regulatory hierarchy where *MaMADS2* has influence over *MaMADS1*, but the reverse seems not to be true, at least in the context of the available data. It is possible that residual *MaMADS1* and *MaMADS2* expression observed in even the most strongly repressed lines contributes to the fact the transgenic bananas eventually reached full ripening, unlike tomatoes harboring the loss-of-function *SlMADS-RIN* gene mutation (*rin*) but similar to heterozygous *rin/RIN* lines used widely in commercial production (Vrebalov et al., 2002). It remains a possibility that the increase in expression of other *MaMADS* box genes observed in MaMADS2-repressed lines, especially *MaMADS1* but possibly others (*MaMADS4*, *MaMADS5*, *MaMADS10*, and/or *MaMADS11*; Figs. 1, 6, and 7C), may contribute to the partial ripening observed in these lines. The possible contributions of these genes remain to be determined, but the data here further support an upstream role of *MaMADS2* in normal banana fruit maturation.

Ethylene May Induce Ripening in *MaMADS2*-Suppressed Lines by Affecting Additional Banana MADS Box Genes

The induction by ethylene of several *SEP MADS* box genes (*MaMADS1* and *MaMADS4*) and *AG* (*MaMADS9*) in the AS *MaMADS2*-suppressed line (Fig. 7C) may contribute to the recovery of ripening in response to exogenous ethylene. The substantial elevation in *MaMADS5* mRNA in response to *MaMADS2* repression is interesting, as is its repression by ethylene at harvest in these same lines (Fig. 7C). MaMADS5 is most similar to banana MaMADS7 (88% identity), which accelerates ripening when expressed in tomato (Liu et al., 2015a). *MaMADS5* and *MaMADS7* are most similar to tomato *SlTAGL11*, which is expressed in fruit (Busi et al., 2003) but for which no phenotype of suppressed lines has been reported. The closest homolog in banana of the tomato ripening gene *SlTAGL1* (Itkin et al., 2009; Vrebalov et al., 2009) is *MaMADS9* (67% identity). In tomato, *SlTAGL1* expression is not influenced by the loss of *SlMADS-RIN* activity in the *rin* mutant (Vrebalov et al., 2009), nor is *MaMADS9* altered when *MaMADS2* is repressed (Fig. 7C). Nevertheless, while *MaMADS9* expression is induced by ethylene in the wild type and in the mutant, the *SlTAGL1* transcript is not affected by ethylene (Itkin et al., 2009; Vrebalov et al., 2009), indicating a divergence of precise regulatory signals for these genes in the two species.

Our results suggest the conservation of multiple ripening MADS box gene functions between fleshy fruits of the monocot banana and the dicot tomato. Nevertheless, the functions of specific orthologs likely diverged in the context of differing fruit physiologies and morphologies presented by banana and tomato.

MaMADS1 and MaMADS2 Are Logical Targets for Banana Ripening Control

Taken together, the repression of either *MaMADS1* or *MaMADS2* results in delayed ethylene synthesis and ripening, although the fruit eventually achieve normal ripening based on an assessment of typical industry and market traits. Moreover, suppressed lines remain sensitive to ethylene, making them suitable to the market practice of ethylene treatment to mature green bananas. It is noteworthy that tomato hybrids heterozygous for the *SlMADS-RIN* mutation (*rin*) are prevalent in the marketplace, conveying inhibited ethylene responsiveness and long shelf-life in the absence of costly postharvest inputs. *MaMADS1* and *MaMADS2* repression phenotypes in banana highlight the potential for similar application to an enormous food crop important for developing country food security and international commerce. The functional utility of these genes in such diverse taxa suggests that similar genes will likely be effective targets for fruit storage and shelf-life improvement in numerous crop species.

MATERIALS AND METHODS

Plant Material

Banana (*Musa acuminate*, AAA Cavendish subgroup, Grand Nain) was used for transformation, and the resulting plants were planted in Western Galilee, Israel. Fruit characteristics were determined in parallel for transgenic and nontransformed control plants grown in the same field. Since fruits of transformed plants were harvested at different times, control fruits were harvested together with the transgenic fruits. Fruit were harvested at the commercial stage (75% final cross-sectional filling), and their maturity and developmental stage were evaluated by determining the average of the locular angles of banana fruit and the ratio of peel to pulp in cross sections (Dadzie and Orchard, 1997). Hands of the first, second, or third tiers containing 10 to 30 fruit were separated from the bunch to monitor the harvest, preclimacteric (green), climacteric (color break), and postclimacteric (appearance of brown spot) stages. Following separation, the cut area of the hands was sprayed with 0.1% (w/v) thiobendazole to prevent crown rot decay, and the hands were air dried, packed in perforated polyethylene bags and stored at 20°C and 95% relative humidity. Pulp and peel samples of at least four fruits were taken separately and frozen on consecutive days, up to 35 DAH. A subset of fruits was treated with ethylene for 20 h from 1 to 4 DAH to assess the ethylene response. Ethylene at 1 μL L^{-1} elicited a response; however, in some cases, fruits were treated with 10 μL L^{-1}, and details are given in the figure legends.

Measurements of Ripening Parameters

Ethylene and carbon dioxide production was measured during postharvest at 20°C. These gases were determined by gas chromatography head space gas analysis of individual banana fingers in 2-L sealed glass jars at 20°C as described (Elitzur et al., 2010). Peel color was determined from the surface area of three individual banana fingers using a Minolta CR-300 colorimeter. Firmness was measured in the middle of whole fruits using a Chatillon Force tester (Ametek). TSS of peel and pulp juice, resulting from freezing and thawing of the tissues, was determined using a hand-held HSR-500 refractometer (Atago).

Construction of Plasmids for MaMADS1 and MaMADS2 Repression

Gene sections used for RNAi or AS constructs for *MaMADS1* (EU869307/Ach11P19540) or *MaMADS2* (EU869306/Ach9P01370) are described in Supplemental Figure S3. Table I summarizes the transgenes created. The plasmids and the correct inserted DNA are described in Supplemental Figure S5. All insertions were under the control of the constitutive 35S promoter. Specifically, an AS construct of *MaMADS2* was created by cloning the 303-bp C domain and 3′ UTR spanning nucleotides 520 to 822 of the *MaMADS2* complementary DNA (cDNA; GenBank accession no. EU869306) in reverse orientation into a modified pBIN binary vector, pBIN117 (kindly provided by Rahan Meristem). The plasmid was generated by *Xho*I and *Eco*RI linker insertion at the *Not*I restriction site of the pBIN117 multiple cloning site. Cloning was facilitated by target sequence PCR using forward and reverse primers containing the *Xho*I and *Eco*RI restriction enzymes sites, respectively (for primer sequences, see Supplemental Table S2). The resulting plasmid contains the NPTII gene under NOS promoter control for kanamycin selection.

Gateway technology (Invitrogen) was used for the RNAi constructs. *MaMADS1* from base 333 to 528 (GenBank accession no. EU869307), representing the partial K and C domains, was cloned by site-specific recombination into pHellsgate2 (GenBank accession no. AJ311874), and *MaMADS2* from base 520 to 822 (GenBank accession no. EU869306), spanning the C domain and 3′ UTR, was cloned into pHellsgate8 (GenBank accession no. AF489904), kindly provided by Commonwealth Scientific and Industrial Research Organization Plant Industry. The *MaMADS1* and *MaMADS2* repression sequences were amplified from banana fruit cDNA using the primers described in Supplemental Table S2, which included the attB1/attB2 recombination sites. The resulting PCR products were gel purified using the QIAquick PCR purification kit (Qiagen) and cloned into pDONR221 (Invitrogen; catalog no. 12536-017), using Gateway BP Clonase II Enzyme Mix (Invitrogen; catalog no. 11789-020) mediated by the attB sites of pDONR. A second Clonase step used LR Clonase II Enzyme Mix (Invitrogen; catalog no. 11791-020) mediated by the attL sites created on pDONR and the attP or attR sites of the entry vector, which exist in pHellsgate2 and pHellsgate8, respectively (Supplemental Fig. S5). The plasmids were verified by restriction enzyme digestion, PCR, and sequencing of PCR products from each plasmid.

Following transformation, the presence of the constructs was verified by PCR on leaf DNA using the Extract-N-Amp Plant PCR Kit (Sigma-Aldrich). The reactions and the expected products are described in Supplemental Figure S5, with primers listed in Supplemental Table S3 and resulting PCR product images shown in Supplemental Figure S5C. Negative control fruit contained no plasmid sequences.

Transformation of Embryonic Banana Cultures

Immature male flowers, approximately 3 d postemergence, were used for the generation of embryonic callus. Male flowers were harvested and treated as described (Strosse et al., 2003). Resulting calli were transferred to an embryonic cell suspension (Schoofs, 1997). Cell clumps were used for transformation by *Agrobacterium tumefaciens* with the inclusion of kanamycin. Transformed somatic embryos were transferred to a semisolid medium containing one-half-strength Murashige and Skoog medium and 10 μM zeatine for approximately 6 months until shoots were clearly visible. Plantlets were hardened under mist in a greenhouse for approximately 6 weeks, until the fourth leaflet emerged, and then transferred to the field (Table I).

Determination of Gene Expression by Q-RT-PCR Analysis

Total RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and treated with TURBO DNase free (Applied Biosystems) as described by the manufacturer. First-strand DNA was prepared using the Verso cDNA Kit (Thermo Fisher Scientific) and used for Q-RT-PCR analysis. Primers (Supplemental Table S4) were designed with Primer Express version 2 (Applied Biosystems). Primer concentration was 4 to 8 μM, and the cDNA was diluted for PCR of the gene of interest and reference *RIBOSOMAL RNA* (*rRNA*) and *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) genes (GenBank accession nos. EU433925 and AY821550, respectively). Template concentrations were determined as described (http://www.abgene.com/downloads/article-SYBRoptimise.pdf.). Forward and reverse primers for the references genes are 5′-GCAAGGATGCCCCAATGT-3′ and 5′-AGCAAGACAGTTGGTTGTGCAG-3′ for *GAPDH* and 5′-GCGACGCATCATTCAAATTTC-3′ and 5′-TCCGGAATCGAACCCTAATTC-3′ for *rRNA*. Reaction mixtures contained cDNA, forward and reverse primers, and Power SYBR Green PCR Master Mix (Applied Biosystems) in a 20-μL total volume. Reactions were run in triplicate on a Rotor-Gene 3000 PCR machine (Corbett Life Research) using 35 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. Results were analyzed with Rotor-Gene 6 and qBase quantification software (http://medgen.ugent.be/qbase/). The data are expressed according to the delta-delta-Ct method according to com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf, and results represent one experiment out of at least two independent samplings, for which two preparations of cDNA were examined.

Analysis of Fruit MaMADS Box Gene Expression

Transcriptome analysis was performed on samples of fruits from four different developmental stages: harvest, prebreaker, breaker, and ripe. RNA was extracted as described above. Handling of samples for RNA sequencing has been described (Fei et al., 2004), and levels of expression are expressed as RPKM. From these data, we selected, for further expression analysis, *MaMADS* box genes with high homology to known genes in tomato (*Solanum lycopersicum*) affecting ripening, which also show expression above 50 RPKM in the pulp.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: GSMUSA Achr10P21480, MaMADS9; GSMUSA Achr5P16870, MaMADS5 (MuMADS1); GSMUSA Achr10P10990, MaMADS7; GSMUSA Achr2P13650, MaMADS10; GSMUSA Achr10P18600, MaMADS11; GSMUSA Achr11P19540, MaMADS1; GSMUSA Achr9P01370, MaMADS2; GSMUSA Achr9P29310, MaMADS8; GSMUSA Achr6P06040, MaMADS4; GSMUSA Achr2T10250, MaMADS3; Solyc07g055920, SlTAGL1; Solyc11g028020, SlTAGL11; Solyc03g114830, SlFUL2; Solyc06g069430, SlFUL1; Solyc05g012020, SlRIN.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Ethylene and carbon dioxide production during ripening in pulp and peel.

Supplemental Figure S2. Highly expressed paralogs of MaMADS box banana fruit genes.

Supplemental Figure S3. Description of the gene segments used to construct the three types of vectors.

Supplemental Figure S4. Ripening parameters of control and knockdown MaMADS1 and MaMADS2 banana fruits following ethylene treatment.

Supplemental Figure S5. Verification of inserts in the various transgenic lines.

Supplemental Table S1. Characterization of transgenic and nontransformed fruit.

Supplemental Table S2. Primers used for the creation of constructs.

Supplemental Table S3. Primers used for the verification of transgenic plants.

Supplemental Table S4. Primers used for the determination of MaMADS transcript levels by Q-RT-PCR.

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