BARD Project Number: IS-4371-10C

Date of submission of the report: 30, October 2014

Project title: Elucidating the Ripening Signaling Pathway in Banana for Improved Fruit Quality, Shelf-life and Food Security

Investigators:

Haya Friedman _____

Julia Vrebalov _____

Jim Giovannoni _____

Affiliated Institutions:

ARO, Volcani Center _____

Boyce Thompson Institute _____

Boyce Thompson Institute _____

Keywords: MaMADS-box genes, ethylene, hormonal regulation, ethylene, auxin, gibberellins, abscisic acid, jasmonic acid, salicylic acid, cytoskeleton

Abbreviations commonly used: abscisic acid (ABA), aminocyclopropane-1-carboxylic acid (ACC), ACC oxidase (ACO), ACC synthase (ACS), AGAMOUS (AG), auxin (IAA), breaker (B), Constitutive Triple Response (CTR), gibberellins (GA), harvest (H), Homeobox (HB), jasmonic acid (JA), Musa acuminata (Ma), MCM1, Agamous, Deficiens, SRF, domains (MADS-box), prebreaker (PB), RIPENING-INHIBITOR (RIN), salicylic acid (SA), ripe (R), RNA interference (RNAi), *SEPALLATA (SEP)*, tomato *AGAMOUS-LIKE1 (TAGL1)*

Budget: IS: \$145,000

US: \$135,000

Total: \$ 280,000

Haya Friedman

Signature

Principal Investigator (PI)

Signature

Institution's Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged		1		
Submitted, in review, in preparation	2			2
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	4			4
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Esther Yakir
Dror Hilman

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	3	1	5
Longer Visits (Sabbaticals)				

Description Cooperation:

The consortium is highly synergistic with an effective team for banana physiology, metabolite analysis and bioinformatics in Israel while transcriptome and additional molecular activities and tomato transformation/analyses are focused on in the US. We are jointly preparing a manuscript that summarizes the phenotypes of the transgenic banana plants. A second paper on whole genome expression analysis is also being prepared (see appendix) based on data accumulated during this project. The Israeli group sent to BTI/Cornell the RNA from the transgenic and WT for transcriptome analysis and the BTI group performed RNA-seq analysis for which the Israeli team performed the statistical analysis and the data mining. The transformation of tomato was also of joint effort where the BTI team performed the transformation and analysis and supplied the plasmids and the Israeli team constructed the vectors.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)			2	2
Licensed

Abstract

Background : Banana being a monocot and having distinct peel and pulp tissues is unique among the fleshy fruits and hence can provide a more comprehensive understanding of fruit ripening. Our previous research which translated ripening discoveries from tomato, led to the identification of six banana fruit-associated MADS-box genes, and we confirmed the positive role of *MaMADS1/2* in banana ripening. The overall goal was to further elucidate the banana ripening signaling pathway as mediated by MADS-box transcriptional regulators. **Specific objectives were:** 1) characterize transcriptional profiles and quality of *MaMADS1/2* repressed fruit; 2) reveal the role of additional *MaMADS* genes in ripening; 3) develop a model of fruit *MaMADS*-box mode of action; and 4) isolate new components of the banana ripening signaling pathway.

Major conclusion: The functions of the banana *MaMADS1-5* have been examined by complementing the *rin* or the *TAGL1*-suppressed lines of tomato. Only *MaMADS5* exhibited partial complementation of *TAGL1*-suppressed and *rin* lines, suggesting that while similar genes play corresponding roles in ripening, evolutionary divergence makes heterologous complementation studies challenging. Nevertheless, the partial complementation of tomato *TAGL1*-suppressed and *rin* lines with *MaMADS5* suggests this gene is likely an important ripening regulator in banana, worthy of further study. RNA-seq transcriptome analysis during ripening was performed on WT and *MaMADS2*-suppressed lines revealing additional candidate genes contributing to ripening control mechanisms. In summary, we discovered 39 *MaMADS*-box genes in addition to homologues of *CNR*, *NOR* and *HB-1* expressed in banana fruits, and which were shown in tomato to play necessary roles in ripening. For most of these genes the expression in peel and pulp was similar. However, a number of key genes were differentially expressed between these tissues indicating that the regulatory components which are active in peel and pulp include both common and tissue-specific regulatory systems, a distinction as compared to the more uniform tomato fruit pericarp.

Because plant hormones are well documented to affect fruit ripening, the expressions of genes within the auxin, gibberellin, abscisic acid, jasmonic acid, salicylic and ethylene signal transduction and synthesis pathways were targeted in our transcriptome analysis. Genes' expression associated with these pathways generally declined during normal ripening in both peel and pulp, excluding cytokinin and ethylene, and this decline was delayed in *MaMADS2*-suppressed banana lines. Hence, we suggest that normal *MaMADS2* activity promotes the observed downward expression within these non-ethylene pathways (especially in the pulp), thus enabling ripening progression. In contrast, the expressions of *ACS* and *ACO* of the ethylene biosynthesis pathway increase in peel and pulp during ripening and are delayed/inhibited in the transgenic bananas, explaining the reduced ethylene production of *MaMADS2*-suppressed lines. Inferred by the different genes' expression in peel and pulp of the gibberellins, salicylic acid and cytokinins pathways, it is suggested that hormonal regulation in these tissues is diverse. These results provide important insights into possible avenues of ripening control in the diverse fruit tissues of banana which was not previously revealed in other ripening systems. As such, our transcriptome analysis of WT and ripening delayed banana mutants provides a starting point for further characterization of ripening. In this study we also developed novel evidence that the cytoskeleton may have a positive role in ripening as components of this pathway were down-regulated by *MaMADS2* suppression. The mode of cytoskeleton involvement in fruit ripening remains unclear but presents a novel new frontier in ripening investigations. In summary, this project yielded functional understanding of the role and mode of action of *MaMADS2* during ripening, pointing to both induction of ethylene and suppression of non-ethylene hormonal signaling pathways. Furthermore, our data suggest important roles for cytoskeleton components and *MaMADS5* in the overall banana ripening control network.

Implications: The project revealed new molecular components/genes involved in banana ripening and refines our understanding of ripening responses in the peel and pulp tissues of this important species. This information is novel as compared to that derived from the more uniform carpel tissues of other highly studied ripening systems including tomato and grape. The work provides specific target genes for potential modification through genetic engineering or for exploration of useful genetic diversity in traditional breeding. The results from the project might point toward improved methods or new treatments to improve banana fruit storage and quality.

Research achievements

Investigations into ripening control in banana, using tomato as a model, to facilitate faster progress has proven both informative and challenging. Studies in tomato demonstrated that in addition to the single SEP-MADS (*SIRIN*) there is an AGAMOUS clade MADS-box gene (*TAGL1*) which is necessary for tomato ripening. Most interestingly, we have shown that SEP-clade MADS-box genes from banana are indeed key regulators for ripening control as they are in tomato (specifically as defined by the tomato *SIMADS-RIN* gene). In contrast to tomato, where a single SEP-MADS regulates ripening (*RIN*), banana utilizes at least two of these genes, *MaMADS1* and *MaMADS2*. We additionally transformed *rin/rin* mutant and *TAGL1RNAi* tomato lines with banana MaMADS1-5 genes. The SEP banana homologs (e.g. *MaMADS1* and *MaMADS2*), however, proved too divergent to function effectively in tomato. Nevertheless, *MaMADS5* gave partial complementation phenotypes in *TAGL1*-repressed and *rin* tomato, suggestive of fruit development and ripening functions, indicating that the heterologous tomato system can be useful in narrowing candidates for a difficult species to transform and regenerate such as banana. In addition to being useful as a heterologous system for addressing banana ripening gene function, tomato has proven exceptionally useful as a source of readily functionally defined genes that can be used to identify banana candidate homologs involved in ripening biology. We identified in the transcriptome data 74 banana MaMADS-box genes including 39 expressed in peel, pulp or both during ripening. Furthermore, a subset of these transcriptional regulatory genes was impacted by MaMADS2 repression suggesting a regulatory cascade of transcription factors and providing candidates for further study. Based on the numbers of MaMADS genes expressed in banana and their complex responses to MaMADS2 repression, the control of ripening in monocot banana is likely more complex than in tomato. This is not surprising given the more complex structure of the banana fruit as compared to other highly studied systems such as tomato and grape. Under the current project we have also initiated analyses of several additional candidate regulatory genes whose fruit expression and sequence (as related to functionally defined tomato homologs) suggest likely roles in ripening regulation. We found banana homologues of tomato NOR, CNR and LHB-1 transcription factors (shown in tomato to be necessary for ripening), that are also expressed in banana fruit and in some cases with differential expression between peel and pulp. These results are in line with the distinct ripening tissues in banana as compared to tomato and clearly demonstrate that ripening control in peel and pulp has both common and distinct components.

Both MaMADS1 and 2 act upstream of climacteric ethylene production. This result makes these transcription factors the first described and functionally defined regulatory proteins operating upstream

of and controlling the ethylene synthesis, so critical to banana fruit ripening biology and production systems. Since the banana transgenic plants are grown in an open field they are exposed to different temperatures. This enabled us to characterize fruit development of the suppressed lines in comparison to WT not only in the context of developmental time but also in response to different temperature regimes. Interestingly, ripening delay due to MaMADS2 suppression was not observed when the field temperature fell below 12°C for an extended period in an unusually cold period occurring during this research. This extreme temperature situation provided an important insight in the context of normally rate limiting transcription factor activities in an unusually cold environment.

The RNA-seq transcriptome profiling of banana peel and pulp through development (harvest (H), pre-breaker (PB), breaker (B) and post-breaker (PB)) fruit stages was analyzed for WT and three independent suppressed lines (MaMADS2 RNAi and antisense; MaMADS1 RNAi). The release of the banana genome sequence has facilitated our efforts as it has both provided a more complete complement of banana candidate genes, and a necessary reference for comprehensive expression profiling. This analysis revealed that the ripening program activated by MaMADS2 in the pulp is different than that in peel. In addition, we have identified that the auxin (IAA), gibberellin (GA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acids (SA) which usually declined at prebreaker of WT are still expressed in the MaMADS2-suppressed lines. This demonstrates that MaMADS2 is responsible for the down regulation of genes within these pathways, as part of the ripening process. As such, our results provide molecular support for reports in the literature suggesting that IAA, GA and SA are negative regulators of fruit ripening. It is more difficult to explain the delay in the expression decline of genes related to ABA and JA, which have been associated in the literature with ripening promotion, though these results help define which hormones place the greatest constraints in the ripening control system. We suggest that the upregulated components within these hormonal pathways function to block ripening. Additional fidelity in the hormonal contributions to distinct fruit tissues were further revealed by the transcriptome analysis. For example, components of the GA pathway are differentially expressed in peel and pulp. Also the expressions of genes within the GA and SA pathways were upregulated in MaMADS2-suppressed lines only in the pulp. Hence, hormonal control related to GA and/or SA is under differential regulatory control in pulp versus peel. The interactions between the hormones IAA, GA, ABA, SA, JA, ethylene and MADS-box genes controlling ripening have not been thoroughly studied and this work provides both basic insights in the context of the diverse fruit tissues of banana, and provides a strong foundation for future investigations that will shed light on ripening control both in banana and in a more general context.

Consistent with the transgenic plants being suppressed in ethylene production, the expression of *ACS* and *ACO* of the ethylene biosynthesis pathway increase in peel and pulp during ripening and are delayed (or inhibited) at least in prebreaker stage. In addition, the decline in expression of *CTR1*, a negative regulator of the ethylene response pathway, (which occurs in WT after harvest) was delayed in the suppressed lines. These observations are consistent with ethylene response inhibition in the MaMADS2-suppressed lines. Interestingly, *CRE1* transcripts, encoding cytokinin receptors, were also prevalent in WT pulp (and reduced in the transgenic mutant) coinciding with ethylene production. Also it was quite clear that while genes within this pathway are expressed in pulp, were hardly expressed in peel, suggesting that CK signalling pathway are active in pulp.

Another interesting and unexpected result of this project is that among the downregulated genes in the *MaMADS2*-suppressed lines Gene Ontologiser (GO) identified a group related to cytoskeleton rearrangements which includes mainly genes of the actin cytoskeleton. The expression of genes within this group increased during ripening and they are maintained at low levels in the MaMADS2 suppressed lines especially at PB. The role of the cytoskeleton in ripening has not yet been investigated. We note that the data obtained for *MaMADS1*-suppressed line was not analyzed as significant suppression of *MaMADS1* transcript was not observed in the tissues examined, although ripening was delayed.

In summary, in this study we generated data and made accomplishments beyond the original scope of the project. Specifically, we have developed whole genome transcriptome profiling data for wild-type fruit development which together with the banana genome sequence enables identification of candidate banana ripening and fruit development genes that will seed further research in our lab and in the labs of others.

Description of cooperation

The consortium is an effective team for banana physiology, metabolite analysis and bioinformatics activities in Israel with molecular activities and tomato transformation/analyses in the US. The Israeli group sent to BTI/Cornell RNA from the transgenic and WT for transcriptome analysis and the BTI group performed the RNAseq analysis for which the Israeli team performed the statistical analysis and the data mining. The transformation of tomato was also of joint effort where the BTI team performed the transformation and supplied the plasmids and the Israeli team constructed the vectors. The principals investigators in both countries met during the project to evaluate the results and summarizing it in several manuscripts (see below). The data set following statistical manipulation is available to the team for further comparison of banana with ripening of other fruits.

List of publications

a) **Banana *MaMADS1* and *MaMADS2* transcription factors regulate fruit ripening and shelf-life**
(Appendix: manuscript 1-this manuscript is completed and should be submitted soon)

b) Review : **Molecular and genetic regulation of fruit ripening** Gapper N, McQuinn R, Giovannoni J. *Plant Mol Biol* 2013, **82**(6):575-591.

c) **MaMADS2 repression delays processes related to hormone signalling pathways at prebreaker stage of banana ripening** (Appendix: manuscript 2- a manuscript has been prepared, and should be submitted by the end of the year)

d) Functional analysis of additional SEP-clade in tomato and a candidate banana gene for an AGAMOUS-associated fruit development and ripening function (will be prepared in the future).

We expect manuscripts, still in preparation, to be reasonably high profile, and additionally, we expect that the resulting findings will be incorporated into at least one review article to be written by the PIs following publication of the primary findings.

Appendix

Table of contents:

Manuscript 1:

Banana *MaMADS1* and *MaMADS2* transcription factors regulate fruit ripening and shelf-life
8-38

Manuscript 2:

MaMADS2 repression delays processes related to hormone signalling pathways at prebreaker stage of banana ripening
39-70

Unpublished data briefly summarized
71-81

Banana *MaMADS1* and *MaMADS2* transcription factors regulate fruit ripening and shelf-life

Tomer Elitzur¹, Esther Yakir¹, Anurag Dagar¹, Julia Vrebalov², Eli Khayat³, James J. Giovannoni², Haya Friedman^{1*}

¹Department of Postharvest Science of Fresh Produce, Agricultural Research Organization (ARO), the Volcani Center, Bet Dagan, Israel, ²Boyce Thompson Institute for Plant Research and USDA-ARS Robert W. Holley Center, Tower Road, Cornell Campus, Ithaca NY, USA, ³Rahan Meristem (1998) Ltd., Rosh Hanikra, Western Galilee, Israel.

*Corresponding author:

Dr. Haya Friedman

Dept. of Postharvest Science of Fresh Produce

ARO, the Volcani Center, P.O. Box 6,

Bet Dagan 50250, Israel

Tel: 972-3-9683618, Fax: 972-3-9683622

E-mail: hayafr@agri.gov.il

Date of submission:

No. of words:

No. of Figures: 9

No. of Tables: 1

Supplementary Figures: 4

Supplementary Tables: 5

Abstract

Fresh fruits and vegetables are important sources of necessary nutrients and are especially prone to decay and loss with disproportionate impacts on food and nutritional security in developing countries. Developed countries expend considerable energy and money on postharvest storage, transportation and ripening/senescence control to counteract the effects of maturation that eventually lead to reduced quality and decay. Genetic solutions to postharvest storage and loss could reduce cost and energy inputs while increasing food security. Banana (*Musa acuminata*) is a major fruit crop and a significant component of worldwide food commerce. We have functionally characterized two banana E class (*SEPALLATA4*) MADS-box genes, *MaMADS1* and *MaMADS2*, homologous to the tomato *RIN-MADS* ripening control gene, and whose expression is associated with ripening in the pulp and peel. Transgenic banana plants were created and independent lines carrying the *MaMADS1* (RNAi) or *MaMADS2* (RNAi or antisense) repression constructs exhibited similar ripening delay and extended shelf-life phenotypes including slower color development and softening when compared to control fruits. Nevertheless, total soluble solids were similar in control and in the transgenic fruits when fruit reached similar color. The delay in fruit ripening in the repressed lines was associated with a delay in climacteric respiration and inhibition in synthesis of the ripening hormone ethylene, which in severely repressed lines even was not produced. Repression of *MaMADS2* (RNAi or antisense) was associated with major changes in gene expression related to multiple pathways consistent with the idea that *MaMADS2* is a major regulator of fruit ripening. Fruits of all transgenic repressed lines responded to exogenous ethylene and ripened normally. Existence of multiple paralogs of *MaMADS1* and *MaMADS2*, may explain the response to exogenous ethylene. Our results show that in banana ripening is controlled by at least two members of the *SEPALLATA* MADS-box gene group and either can serve as a target for ripening control in this important fruit crop.

Introduction

Fruits of plants in the genus *Musa* including bananas and plantains represent staple foods for millions of people especially in developing countries and 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 to absent and losses are correspondingly high. Regulating ripening and extending banana shelf life via genetic means will facilitate ambient temperature handling of banana with great benefit for producers and developing country consumers, 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 and genetic ripening mechanisms have been thoroughly studied in this fleshy fruit (Grierson, 2013; Klee and Giovannoni, 2011). Banana and tomato fruit exhibit so-called climacteric respiration (a ripening-associated increase in respiration) and a surge in ethylene production concomitant with ripening (Grierson, 2013; Klee and Giovannoni, 2011; Seymour et al., 2013). Tomato genes controlling ethylene production and climacteric respiration have been identified (Alexander and Grierson, 2002; Giovannoni, 2007; Pirrello et al., 2009). Among them are two MADS-box genes; *SIMADS-RIN* of the *SEPALLATA* E function clade (Ito et al., 2008; Vrebalov et al., 2002) and *TAGLI*, an AGAMOUS-like MADS-box gene, necessary for both early fruit expansion and later ripening (Itkin et al., 2009; Vrebalov et al., 2009); *NOR*, a NAC-domain protein which acts upstream to *RIN* in tomato, in addition to another NAC protein, *SINAC4* (Zhu et al., 2014); SQUAMOSA Promoter Binding Protein *SISPB-CNR* (Manning et al., 2006; Seymour et al., 2008) which interacts with *SIMADS-RIN* to promote *RIN* target gene promoter binding (Martel et al., 2011), *SIEREBP6* and *SIAP2a* that negatively regulate ethylene synthesis and carotenoid accumulation (Chung et al., 2010; Karlova et al., 2011; Lee et al., 2012) and the HD-Zip homeobox protein, *SIHB-1*, which interacts with the promoter of *SIACO1* and *SIACS* (Lin et al., 2008). A Golden-like 2 (GLK2) transcription factor was shown to influence plastid accumulation,

pigmentation, vitamin C and sugar levels of tomato fruit (Nguyen et al., 2014; Powell et al., 2012). Reduction in the expression of these genes either by transgenic manipulation or naturally by mutation altered fruit ripening progression (Giovannoni, 2001, 2004; Vrebalov et al., 2002). For example, either down regulation of *SIMADS-RIN* or a deletion of its c-terminus, 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). This is further supported by the fact that application of exogenous ethylene to *rin/rin* mutant non-ripening fruit does not restore ripening (Giovannoni, 2001; Lincoln and Fischer, 1988; Vrebalov et al., 2002). Indeed, a naturally occurring loss-of-function mutation in the *SIMADS-RIN* gene is the basis of the *rin* mutation which is widely used in hybrid tomato plants the world-over for ripening and shelf-life control.

Further support for *SIMADS-RIN* action as a modulator of ripening, ethylene production and associated downstream ripening phenomena, is its mRNA accumulation profile that increases concomitant with ripening ethylene (Kitagawa et al., 2005; Vrebalov et al., 2002). Moreover, *SIMADS-RIN* homodimers bind to consensus promoter regions of genes involved in ethylene synthesis (Ito et al., 2008), in addition to genes representing key steps in ripening control, i.e. the promoters of *SIMADS-RIN* itself and additional ripening-associated transcription factors, the major fruit pectinase, polygalacturonase (*PG*), fruit-associated ethylene receptors and the rate limiting step in fruit carotenoid accumulation, phytoene synthase 1 (*PSY1*). These interactions are further dependent on normal *CNR* activity (Martel et al., 2011). The *SIMADS-RIN* protein interacts with additional MADS-box proteins including the tomato *FRUITFUL* homologs *FUL1*, *FUL2* and *TAGL1* (Fujisawa et al., 2014; Martel et al., 2011). It has recently been shown that *SIMADS-RIN* promoter binding is associated with fruit development changes in the methylome, and that *RIN* activity is necessary for at least some of these changes (Zhong et al., 2013).

Similar ripening components especially of the MADS-box family were discovered in other fruits (Boss et al., 2002; Rigola et al., 1998; Sung et al., 2001; Tadiello et al., 2009; Yao et al., 1999), including

strawberries a, a so-called non-climacteric fruit which ripen without elevated respiration or ethylene (Seymour et al., 2011). Nevertheless, the function of MADS box genes in ripening of many other important fruit crops remains poorly investigated despite the enormous potential toward improving fruit storage, transport and associated impacts on food security. Toward this end we focused on banana (*Musa acuminata*), a crop of world-wide importance, a major food commodity and a staple crop in areas of Africa and Asia. Banana, additionally, represents the monocot lineage of plants which have not been well characterized at the molecular level in terms of fruit ripening.

We have previously described the peel and pulp temporal expression of six banana fruit-associated *MaMADS*-box genes of which, four belong to the *SEPALLATA* clade (E class *MaMADS1-4*), one to *AGAMOUS* (*MaMADS5*) and another to *PISTELLATA* (*MaMADS6*) (Elitzur et al., 2010). The expression of the *MaMADS1-5* genes was induced during ripening with gene-specific variation in the pulp and peel, and *MaMADS5* was also found to bind to CArG box sequences which exist in the promoters of major ripening genes (Choudhury et al., 2012). Interestingly, an increase in *MaMADS*-box gene expression in the pulp occurred concomitantly with climacteric respiration and preceded the ethylene hormone burst, consistent with a role in ripening regulation similar to that reported in tomato and strawberry (both dicot) MADS-box gene counterparts. This was followed by increased ethylene synthesis and elevated expression of *MaMADS*-box genes in the peel. Many of these genes were ethylene-inducible in peel and/or pulp. Specifically, *MaMADS3*, 4 and 5 are induced by ethylene in the pulp and *MaMADS1* and 3 in the peel, while treatment of fruit with the ethylene response inhibitor 1-MCP increased expression of *MaMADS1* and 4 in pulp, but decreased expression of all genes in peel. *MaMADS1* and 2 are not induced by ethylene in pulp and *MaMADS2* was the only gene expressed independent of ethylene in both peel and pulp, suggesting more primary roles in ripening for these as compared to the other *MaMADS* genes. 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 both are necessary for ripening. This study shows their clear practical utility as genetic

targets for controlling shelf-life and quality in a fruit important to food security and international commerce.

Materials and Methods

Plant material

Banana (*Musa acuminata*, AAA Cavendish subgroup, Grand Nain) was used for transformation and the resulting plants were planted along the Mediterranean northern shore of Israel. Banana fruit of control and transgenic plants were harvested between May and June 2009, and again on October-December 2011. Fruit characteristics were determined in parallel for transgenic and non-transformed typical plants grown in the same field. Since fruits of transformed plants were harvested at different times, two groups of controls are presented one (control I) for transgenic MaMADS1 and another (control II) for transgenic MaMADS2. Fruit were harvested at 75% final cross-sectional filling. Fruit maturity and development was determined 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 10-30 fruit were separated from the bunch to monitor the pre-climacteric (green), climacteric and post-climacteric stages. Following separation, the cut area of the hands was sprayed with 0.1% thiobendazole to prevent crown rot decay, and the hands were air-dried, packed in perforated polyethylene bags and stored at 20°C and 95% RH. Samples were taken from pulp and peel, separately, on consecutive days, up to 35 days after harvest (DAH). Fruit were treated with ethylene (1-10 µl L⁻¹) for 20 h on the first to the third DAH to assess response to ethylene.

Ripening measurements

Ethylene (C₂H₄) and carbon dioxide (CO₂) production were determined by HPLC 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 surface area of three individual banana fingers using a Minolta CR-300

colorimeter (Minolta Corporation, New Jersey, USA). Firmness was measured in the middle of whole fruits using a Chatillon Force tester (Ametek Inc., Florida USA). Total soluble solids (TSS) of peel and pulp juice, resulting from freezing and thawing of the tissues, was determined using a handheld HSR-500 refractometer (Atago Co. Ltd, Japan).

Construction of plasmids for MaMADS1 and MaMADS2 repression

RNAi or antisense constructs for *MaMADS1* (EU869307) or *MaMADS2* (EU869306) are described in Table 1 and the genes' segments are described in Supplementary Fig. S1. All transgenes were under the control of the constitutive 35S promoter. Specifically, an antisense construct of *MaMADS2* was created by cloning the 303 bp C-domain and 3'UTR regions spanning nucleotides 520-823 of the *MaMADS2* cDNA (Genbank accession EU869306), in reverse orientation into a modified pBINbinary vector, pBIN117 (kindly provided by Rahan Meristem Ltd., Israel). The plasmid was generated by XhoI, EcoRI linker insertion at the NotI restriction site of the pBIN117 multiple cloning site. Cloning was facilitated by target sequence PCR using forward (FW) and reverse (RV) primers containing the XhoI and EcoRI restriction enzymes sites, respectively (see Supplementary Table, S1 for primers sequences). The resulting plasmid contains the NPTII gene under NOS promoter control, for Kanamycin selection. Gateway technology (Invitrogen Inc., Carlsbad, Calif.) was used for the RNAi constructs. *MaMADS1* from base 333-641 (Genbank EU869307) representing partial K and partial C domains was cloned by site-specific recombination into pHellsgate2 (Genbank AJ311874) and *MaMADS2* from bases 520-823 (Genbank EU869306) spanning the C-domain and 3'UTR was cloned into pHellsgate8 (Genbank AF489904), kindly provided by CSIRO Plant Industry, Canberra, Australia. *MaMADS1* and *MaMADS2* repression sequences were amplified from banana fruit cDNA using the primers described in Supplementary Table S1 and included the attB1/attB2 recombination sites. The resulting PCR products were gel-purified using the QIAquick PCR purification kit (Qiagen, Maryland, MD, USA) and cloned into pDONR 221 (Invitrogen Carlsbad, CA, USA Cat. No. 12536-017), using Gateway BP Clonase™

II Enzyme Mix (Invitrogen Cat. No. 11789-020) mediated by the attB sites of the pDONR. A second Clonase step using LR Clonase™ II Enzyme Mix (Invitrogen Cat. No. 11791-020) mediated by the attL sites created on the pDONR and the attP or the attR sites of the entry vector which exist in pHellsgate2 and pHellsgate8, respectively (Supplementary Fig. S2A). The plasmids were verified by restriction enzymes digest, PCR and sequencing of PCR products from each plasmid.

Following transformation the existence 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 Supplementary Fig. S2A with primers listed in Supplementary Table, S2 and resulting PCR product images in Supplementary Fig. S2C. Negative control fruit contained no plasmid sequences.

Transformation of embryonic banana cultures

The transformation of the above constructs into banana was performed by Rahan Meristem Ltd. Immature male flowers were used for the generation of embryonic callus. Resulting calli were transferred to embryonic cell suspension (Schoofs, 1997). Cell clumps were used for transformation by *Agrobacterium* with the inclusion of kanamycin. Transformed somatic embryos were transferred to a medium containing half strength MS and 10 μ M zeatine for approximately six months until shoots were clearly visible. Plantlets were hardened in a greenhouse under mist until the 4-leaflet stage (Table 1).

Determination of gene expression by quantitative RT-PCR (Q-RT-PCR) analysis

Total RNA was extracted using the Spectrum kit (Sigma Aldrich) and treated with TURBO™ DNase free (Applied Biosystems, USA) as described by the manufacturer (<https://products.appliedbiosystems.com>). First strand DNA was prepared using the Verso™ cDNA kit (Thermo Fisher Scientific Inc., USA), and used for Q-RT-PCR analysis. Primers (Supplementary Table S3) were designed with Primer Express (v. 2, Applied Biosystems). Primer specificity was described previously (Elitzur et al., 2010). Primers concentration was 4-8 μ M and the cDNA was diluted 1:20 and

1:5000 for PCR of the gene of interest and reference rRNA and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes (Genbank EU433925, AY821550, respectively). Template concentrations were determined as described (<http://www.abgene.com/downloads/article-SYBROptimise.pdf>). Reaction mixtures contained cDNA, forward and reverse primers and Power SYBR Green PCR Master mix (Applied Biosystems, USA) in a 20 µl total 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 sec, 60°C for 15 sec, and 72°C for 20 sec. 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 ribosomal gene. Results were analyzed with Rotor-Gene 6 and qBase quantification Software (<http://medgen.ugent.be/qbase/>). The data is expressed according to the delta-delta-Ct method and results represent one experiment out of at least two independent samplings, for which two to four preparations of cDNA were examined.

Transcriptome analysis

The transcriptome of control, as well as, *MaMADS2* antisense (#44) and *MaMADS2* RNAi (#32) lines was determined by RNAseq analysis. Samples were collected on 2011 from fruits of different developmental stages: harvest (H), pre-breaker (PB), breaker (B) and ripe (R). The stages were determined by the color of the peel using Minolta (see above). The average of hue ° from at least two replicates for control, *MaMADS2* antisense (#44) and *MaMADS2* RNAi (#32), were: H:118, 119.3, 119.3 ; PB:106.3, 113.3, 108; B:100.5, 99.1, 96.1; R: 86.2, 89, 88.4, respectively. In this experiment the color change of *MaMADS2* antisense was delayed by 5 days and that of *MaMADS2* RNAi by 11 days compared to control (data not shown). RNA was extracted as described above and handling of samples for RNAseq is described at and levels of expression were expressed as RPMK.

Since most of the physiological changes in the repressed lines occurred prior to breaker, analysis of total transcripts' up and down regulated was performed on fold change between B to H. A Venn diagram was performed with VENNY as described (Oliveros, 2007), to display the number of genes up or down regulated in control and repressed lines.

Further analysis was performed to describe the major patterns of genes' expression during fruit ripening. The maximum and the minimum transcript levels in WT of each of the genes were used to calculate a ratio exhibiting expression modification during fruit ripening. Out of the 36,538 banana genes, 16,408 with a ratio of more than 2 and less than 100 were chosen for cluster analysis. For each of these genes the averages of fold change of PB to H (PB-H), B to PB (PB-B) and R to B (B-R) were calculated, and clustering analysis was performed with EXPANDER (EXpression Analyzer and DisplayER) (<http://acgt.cs.tau.ac.il/expander/>) and Click clustering algorithm (Sharan et al., 2003). Click clustering algorithm grouped the genes into 13 different clusters and they were joined together into four clusters based on expression similarity. The expression patterns of the same genes were calculated for each of the stages of the repressed lines, and the results were plotted with Multiple Experiment Viewer (MeV) <http://sourceforge.net/p/mev-tm4/discussion/>. Each of the WT clusters was analyzed by Ontologizer (Parent-Child-Union, Benjamini-Hochberg) to detect enrichment of GO (Gene Ontology) <http://compbio.charite.de/index.php/ontologizer2.html>. GO with FDR<0.05 were grouped into biological pathways as described in Supplementary Table S5.

Results

Transgenic banana plants with reduced expression of MaMADS1 or MaMADS2

We previously described the isolation of 6 full length MaMADS-box genes from banana (Elitzur et al., 2010). Here we functionally define two (MaMADS1 and MaMADS2) whose expression is elevated in concert with ripening, but was not affected by ethylene in the pulp. These genes show high peptide

similarity to each other (especially in their M domains) with the C-region most divergent (Elitzur et al., 2010). Three repression constructs targeting different gene sections were created: a) RNAi MaMADS1 targets the K and C domains ; b) RNAi MaMADS2 targets the C domain and the 3'UTR; c) Antisense MaMADS2 targets the same sequence described for b. The constructs are depicted in Supplementary Fig. S1, and detailed in 'Materials and Methods'. The constructs were used for transformation of embryo cultures and transgenic plants recovered as described in Table 1. The transformation of all three constructs yielded fertile plants verified for inserts using leaf DNA as described in 'Materials and Methods' (see also Supplementary Fig. S2).

Banana fruit were harvested from independent transgene-confirmed trees of all three transgenic classes; RNAi *MaMADS1*, RNAi *MaMADS2*, and Antisense *MaMADS2*, in addition to control plants. Target gene mRNA levels at breaker (color transition) stage in peel and pulp were determined by quantitative PCR (qPCR; Fig. 1). *MaMADS1* and *MaMADS2* mRNA accumulation in control plants was high in peel and pulp, as expected from prior results (Elitzur et al., 2010), but the *MaMADS1* mRNA was low in RNAi *MaMADS1*, and *MaMADS2* mRNA was low in RNAi *MaMADS2* and antisense *MaMADS2* peel and pulp for all transgenic plants examined (Fig. 1).

Ripening characterization of MaMADS1 and MaMADS2 repressed banana fruit

In order to ascertain the impact of MaMADS1 or MaMADS2 repression on ripening, we monitored transgenic and control fruit. Transgenic and control fruit and hands (fruit clusters) are shown in Fig. 2 where it is clear that at 19-20 days postharvest the control fruit show severe decay (Fig. 2 A, C) while transgenic fruit remain without damage even at 26 days (Fig. 2 B, D) following harvest. This result demonstrates a strong influence on ripening and shelflife of the MaMADS1 and MaMADS2 transcription factors.

To further define the effects of the banana MADS genes on ripening, respiration and ethylene production was measured on control and transgenic fruit on consecutive days from harvest until the

appearance of brown spots (Fig. 3). In each of the fruit in the Control I group, the climacteric respiration increase (9-12 DAH) preceded the burst in ethylene production (12 - 15 DAH) as expected (Fig. 3A). Fruit of two independently transformed RNAi *MaMADS1* plants were analyzed, and in one (RNAi-*MaMADS1*-20), no ethylene peak was detected and only slightly increased respiration occurred at 21 DAH. In fruit from the second (RNAi-*MaMADS1*-19), a delayed respiration burst occurred at 14th DAH followed by an attenuated ethylene peak at 19th DAH (Fig. 3B). These observations confirm a regulatory role of *MaMADS1* in ripening via control of climacteric respiration and ethylene hormone synthesis.

Among the Control II group, increase in ethylene production preceded the respiration peak and occurred 10-11 DAH, while respiration peaked at 15 DAH (Fig. 3C). In comparison, the ethylene peak in fruit from RNAi-*MaMADS2* transgenic plants was lower than in control fruit and appeared on the 16th DAH in parallel with increased respiration (highest respiration occurred between the 14-20th DAH in fruit from the three different plants) (Fig. 3D). Greater inhibition of ethylene production and respiration was observed in banana fruit from antisense *MaMADS2* transgenic plants (Fig. 3E). In two plants (AS 40 and AS 45) the banana fruit did not produce any ethylene and in these fruits respiration increased gradually starting from the 26 th DAH. In two other plants the ethylene production peak was lower than that of controls, and occurred on the 16 th (AS 37) or on the 19 th (AS 36) DAH. The peaks in these two types of fruit occurred in parallel with increased respiration. Note that only in two antisense *MaMADS2* fruit which did not exhibit ethylene production, respiration increased gradually, while in all other fruit respiration increased concomitantly with the ethylene peak. Few of the transgenic lines, in addition to other lines of the same transformation were reexamined on 2011, and the ripening of their fruits was also delayed (data not shown).

The development of plant chimeras with and without the transgene within fruit bunches further corroborated the involvement of *MaMADS1* in fruit ripening. Besides the banana bunches used for the above study, some of the bunches were not uniform in their color development after harvest and bananas

derived from the fifth hand exhibited earlier ripening than banana from upper (older) hands (Fig. 4A). To further elucidate this phenomenon, the DNA of banana from different hands of the same bunch of three different bunches were examined (Fig. 4B). It can be seen that in three plants that harbor the correct insert (as judged by leaf PCR), no inserts was observed in the fifth hand, and only in two bunches an insert was observed in the 2ed hand, suggesting the transformation yielded chimera plants. These results were in agreement with ethylene and carbon dioxide production (Fig. 4C). In control fruits the ethylene production peak occurred on the 11th day after harvest in bananas from the second and the fifth hand, similar to banana which did not harbor the insert. However, the ethylene and carbon dioxide peaks appeared 7 days later in fruits that harbor the insert (plants 8 and 18 2ed hand).

The parameters of color, firmness and TSS were followed in the various fruit of control and transgenic plants (Fig. 5, Supplementary Table, S4). 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, 5), while in transgenic plants, especially those that did not produce ethylene, it occurred either following or concomitantly with the increase in respiration. At this stage usually fruits of transgenic plants had higher firmness than control. The TSS at the peel and at the pulp irrespective of the type of transgenic, were in some lines lower than control and in others very similar.

Response of MaMADS1 or MaMADS2 repressed banana fruit to exogenous ethylene

It was clear that normal ripening was delayed in fruit of the transgenic plants, and hence it was important to determine if these fruit still exhibited ripening delay following ethylene treatment. Banana fruit of the control and transgenic plants were exposed to ethylene (Fig. 6) and it can be seen that fruit of the transgenic plants responded to ethylene in a similar manner to that of control fruit and developed yellow color. Examination of firmness and color showed that there was no difference between controls and any of the transgenic plants following ethylene treatment (Supplementary Fig. S3).

Analysis of MaMADS-box genes' expression in the fruit of MaMADS1 and MaMADS2 repressed lines

To examine the specificity of suppression, we have determined the levels of expression in *MaMADS1* and *MaMADS2* repressed lines of the reciprocal genes (Fig. 7), in addition to *MaMADS3-5* expression (Fig. 8). The expression levels of *MaMADS2* in control plants was similar between peel and pulp (Fig. 7A, and also Fig. 1B, C), and the levels of *MaMADS1* was higher in peel than in pulp (Fig. 7B and also Fig. 1A) similar to published results (Elitzur et al., 2010). Downregulation of *MaMADS1* did not reduce the levels of *MaMADS2* in the pulp, but did reduce it in the peel (Fig. 7A), suggesting a regulatory rather than transgene effect. This probably occurred due to the lack of tight homology in the sequences used for repression (Supplementary Fig. S1). On the other hand, downregulation of *MaMADS2* (either by antisense or RNAi) induced *MaMADS1* in the pulp but reduced it in the peel, (Fig. 7B, C). Again, these results are consistent with regulatory as opposed to unintended direct transgene effects.

To study the possible interactions between *MaMADS1* or *MaMADS2* and *MaMADS3-5*, the expression of *MaMADS3*, 4 and 5 have been determined in *MaMADS1* (Fig. 7A) or *MaMADS2* repressed lines (Fig. 8B, C). The levels of *MaMADS3* which was higher in peel than in pulp, was reduced in both types of transgenic plants in comparison to control fruit. On the other hand, the levels of either *MaMADS4* or *MaMADS5* increased in the pulp of *MaMADS2* and *MaMADS1* knockdown plants. Similar results were also observed for the *MaMADS2* repressed fruits taken at 2011 for RNAseq analysis (data not shown). These results indicate that *MaMADS4* or *MaMADS5* genes are under a negative control of *MaMADS2* and *MaMADS1* in the pulp.

Transcriptome analysis of WT and MaMADS2 repressed fruits during ripening.

RNAseq data was obtained from four stages of fruit development: harvest (H), pre breaker (PB), breaker (B) and ripe (R) of control and *MaMADS2* repressed fruits. Since the delay in ripening in the mutant banana fruit was observed mostly prior to breaker (Fig. 5), the change in gene expression from harvest to breaker was examined in control and in *MaMADS2* repressed lines (antisense and RNAi)

(Supplementary Fig. 4). In general, at the transition from harvest to breaker more genes were downregulated than upregulated in peel and pulp in control, as well as in the mutants (i.e., in WT there are 7124 , 5912 downregulated genes in peel in pulp, respectively; and 1879, 2452 upregulated genes in peel and pulp, respectively). The largest difference in the number of modified genes from harvest to breaker between the mutants and the control was observed for the up-regulated genes in the pulp. The ratios between number of modified genes in *MaMADS2*-RNAi to WT were 1.95 and 1.36 in pulp and peel, respectively. In comparison, the ratios for down regulated genes were 1.11 for pulp and pulp and 1.06 for peel. Similar trend was observed for ratios between *MaMADS2* antisense to WT. These indicate that the major effect of *MaMADS2* expression is in the pulp, and *MaMADS2* is acting to modulate mainly upregulated genes.

Patterns of genes' expression between the different developmental stages for H-PB, PB-B, R-B were grouped into 4 expression clusters (Fig. 9A), which were subjected to GO analysis (Fig. 9A and Supplementary Table S5). Clusters 1 and 3 were mostly of downregulated and 2 and 4 of upregulated genes at the initial stage (H-PB). Cluster 1 is enriched in gene ontology related to mitochondria, membrane envelope and ribosome and translation which coincided with increase in GO related to gene expression. This cluster was also enriched in GO related to response to stimuli which is composed in response to different hormones. Cluster 3 which is characterized by reduced expression at early and late stages was also enriched in membrane envelope, but also in cell wall, cytoskeleton and plastids related genes. In addition , metabolism of phosphorous, sulfur, amino acids and carbohydrate GO functions were also enriched. Cluster 2 was enriched in GO functions of chromatin silencing and protein modification and also of Golgi and cytoskeleton which are related to the GO of calcium binding and transport. GO function of carbohydrate metabolism is also enriched in this cluster. Cluster 4 which consists of genes induced at early and late stages was enriched in GO functions related to RNA metabolism and protein degradation and also of metabolism of phosphorus and nucleotides.

The expression patterns of the same genes in the two types of transgenic plants are displayed, and it is clear that the pattern of expression is completely different. This occurs despite the fact that control and mutant samples were in the same physiological stage based on color of the peel. The results suggest that *MADS2* is responsible for the execution of the ripening program.

Discussion

Much of our knowledge on 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 components and mechanisms. In this study we have established in banana that two of the fruit class E *SEPALATTA* *MaMADS-box* genes are positive regulators of ripening. Reducing the transcript levels of either of these two genes (Fig. 1), decreased ripening progression, as was demonstrated by inhibition in color change, delayed softening and slower accumulation of sugar in the pulp and peel (Figs. 2, 3, 4, 5). The fruits of the transgenic, once ripened, had similar TSS, but were firmer in comparison to control fruits which achieved similar stage few days earlier (Fig. 5, Supplementary Table, S4).

Silencing each of the genes either delayed and reduced ethylene production or completely abolished it. However, the climacteric respiration as determined by levels of carbon dioxide, although it was delayed in *MaMADS1* and *MaMADS2* suppressed fruits, ultimately reached to that in control fruit. Delayed climacteric respiration appeared even in fruits that did not produce any ethylene either from *MaMADS1* RNAi or from *MaMADS2* antisense. This indicates that although increase in respiration might be affected by either *MaMADS1* or *MaMADS2*, it is not totally dependent on either of these genes, and in their absence, other gene(s) might lead to induced respiration.

In our previous study we hypothesized that *MaMADS2* has a central role in ripening since its expression was not modified in peel and pulp by ethylene (Elitzur et al., 2010). A support for this idea is seen in the transcriptome data showing that there are major changes in genes expression related to ripening in the repressed line (Fig. 9). Moreover, *MaMADS2* exert its effect mainly on the upregulated genes in the

pulp, consistent with the idea that it is responsible to down regulate negative modulators of ripening. Also, MaMADS2 negatively regulate the expression of *MaMADS1*, 4 and 5 in pulp and positively regulate *MaMADS3* gene expression in the peel, (Figs. 7, 8). However, in comparison, the levels of *MaMADS2* expression in pulp were not affected in *MaMADS1* repressed fruit. Nevertheless, it seems that MaMADS1 negatively controls *MaMADS5* and *MaMADS4* genes expression in pulp and positively that of *MaMADS3* in the peel, where this gene is mainly expressed. The increase in expression of other negatively regulated *MaMADS* genes might explain why there is no complete inhibition of ripening. It is possible that with neither MaMADS1 nor MaMADS2, other genes execute the ripening process.

While *MaMADS1-4* genes belong to the *SEP* clade, *MaMADS5* belongs to the *AGAMOUS* (Choudhury et al., 2012; Elitzur et al., 2010). *MaMADS5* was also found to be constitutively expressed in the peel but was induced in the pulp concomitantly with the increase in respiration. In addition, the expression of *MaMADS5* is negatively controlled in the pulp by both MaMADS1 and MaMADS2. All these indicate that MaMADS5 serves a unique function in ripening. An *AGAMOUS* gene *TAGL1* was discovered in tomato which play a role in fruit ripening and growth (Itkin et al., 2009; Vrebalov et al., 2009). Also the protein of MaMADS5 was found to bind in banana to consensus CArG, which are found in ripening-related genes. Hence, it is possible that also in banana MaMADS5 fulfill a similar function.

The control of ripening is indeed executed in banana by using similar components to those in tomato and with generally similar effect; however, the number and activities of these components differ from tomato possibly in part due to differences in fruit morphology. One major difference is that while tomato fruit lacking the *SIMADS-RIN* do not ripen upon ethylene addition, fruit of each of the banana transgenic plants ripen normally as a result of ethylene application, possibly due to lack of complete inhibition (Fig. 6, Supplementary Fig. S3). In addition, it is possible that the multiple MaMADS-box genes of which their expression levels is induced by ethylene, might substitute for the lack of each of the genes and lead to induction of the climacteric response.

In conclusion, we have isolated and functionally characterized two banana E class *MADS-box* genes whose expressions are associated with ripening, but which were not necessary for ethylene response, suggesting dominant ripening regulatory functions similar to the tomato *SEPETALA* clade gene *SIMADS-RIN*. Repression of either, results in delayed ethylene synthesis and ripening, though at later dates the fruit achieve apparently normal ripening features. This suggests that MADS-box ripening function is an ancient activity of this gene family (as it is common to both mono- and dicots). It is noteworthy that tomato hybrids heterozygous for the *SIMADS-RIN rin* mutation are prevalent in the marketplace with long-shelf life and reduced ethylene responsiveness. MaMADS1 and 2 clearly serve similar functions and with potential application to an enormous food crop important for developing country food security and international commerce.

Figures

Fig. 1: Determination of *MaMADS1* and *MaMADS2* expression levels in their corresponding knock-down transgenic plants. *MaMADS1* expression (A) was determined in two *MaMADS1* RNAi transgenic plants and *MaMADS2* expression (B, C) was determined in RNAi transgenic plants of *MaMADS2* (B) and in transgenic plants of antisense *MaMADS2* (C). The expression was determined in peel and pulp of fruit at breaker stage. Banana fruits of first hand were used in A and of the third hand in B and C. Sampling times (DAH) were: for control fruit in A-12 d (con 1) and 14 d (con 2), and in B and C- 10 d; for *MaMADS1* RNAi (A) - 19 d (plant 19) and 23 d (plant 20); for *MaMADS2* RNAi (B) - 16 d (plants 21,23,24;) and for antisense *MaMADS2* (C) – 19 d (plant 36), 16 d (plant 37) and 24 d plants 40,45). Primers used for the expression analysis are described in Supplementary Table, S3.

Fig. 2: Banana fruits of control and knock-down *MaMADS1* and *MaMADS2* transgenic plants. Individual banana fruit are presented from each of the transgenic antisense *MaMADS2* and RNAi *MaMADS2* and from control plants (A, B), and whole hands from RNAi *MaMADS1* and control plants (C, D). Fruits of the first hand were photographed at 19 DAH (A), 26 DAH (B), and of the second hands at 20 DAH (C) and 26 DAH (D).

Fig. 3: Comparison of ethylene and CO_2 production after harvest between control and knock-down *MaMADS1* and *MaMADS2* fruits. Production was determined for control fruits (A, C) at two independent experiments; Control I on May 2009 (A) and Control II on June 2009 (C). Control I fruit

served as control for transgenic RNAi *MaMADS1* (B) and Control II for either RNAi *MaMADS2* (D) or antisense *MaMADS2* (E). Measurement for control I and RNAi *MaMADS1* banana was performed on fruit of the third hand and that for Control II and RNAi *MaMADS2* (D) or antisense *MaMADS2* (E) on fruit the first hand.

Fig. 4: Description of chimera obtained from transgenic plants harboring the RNAi-*MaMADS1* constructs. (A)-Photographs of fruit from hand 1, 2 and 5 of plant 2-18; (B)- Confirmation of insert in DNA extracted from leaves of three independent banana plants (2-8, 2-18, 2-21) in comparison to Control non-transgenic plants. The primers used for confirmation are described in Supplementary Table, S2 (reaction a); (C)-Ethylene (left panel) and carbon dioxide (right panel) productions in hands 2 and 5 taken from control and transgenic plants. L-leaf; H-fruit from the indicated hand; P-positive plasmid; Con- non-transgenic banana plant. Numbers above lanes in B indicate the hand number.

Fig. 5: Quality parameters of repressed *MaMADS1* and *MaMADS2* banana fruit. The parameters of color (h°), firmness (N), and TSS (Brix (%)) in peel and pulp were examined after harvest in RNAi-*MaMADS1* third hand (A) and RNAi(B) /antisense (C) *MaMADS2* first hand and in the corresponding control plants. Summary of parameters is described in Supplementary Table, S4.

Fig. 6: Response to ethylene of banana fruits from control and knockdown *MaMADS1* and *MaMADS2* transgenic plants. Ethylene-treated (E) and non-treated (C) fruit of control (A), *MaMADS1* RNAi (B), *MaMADS2* RNAi (C) and *MaMADS2* antisense (D) are displayed. Fruits were treated with ethylene (10 μ L/L for B or 1 μ L/L for A, C, D) immediately after harvest for one day and photographed following 12 days for control plants (A), 9 days for RNAi *MaMADS1* (B) and 12 days for *MaMADS2* RNAi (C)/antisense (D). Summary of quality parameters are displayed in Supplementary Fig. S3.

Fig. 7: Determination of expression levels of *MaMADS2* in *MaMADS1* repressed and *MaMADS1* in *MaMADS2* repressed lines. *MaMADS2* expression (A) was determined in two *MaMADS1* RNAi transgenic plants and *MaMADS1* expression (B, C) was determined in RNAi transgenic plants of *MaMADS2* (B) and in transgenic of antisense *MaMADS2* (C). Samples for analysis were obtained as described in Fig. 1. Primers used for the expression analysis are described in Supplementary Table, S3.

Fig. 8: Expression patterns of *MaMADS3*, *MaMADS4* and *MaMADS5* in repressed *MaMADS1* and *MaMADS2* fruits. Expression was determined in *MaMADS1* RNAi and *MaMADS2* RNAi/antisense transgenic plants at samples obtained as described in Fig. 1. Primers used for the expression analysis are described in Supplementary Table, S3.

Fig. 9: Cluster and functional analysis of genes' expression in WT and a comparison to expression in transgenic lines of *MaMADS2* RNAi and *MaMADS2* antisense. Data of RNA-seq was obtained from pulp of harvest (H), pre-breaker (PB), breaker (B) and ripe (R) samples and the averages of fold change in WT pulp was calculated between the stages (H-PB, PB-B and B-R). Genes (16,408) of max/min expression of more than 2 and less than 100 were categorized into 4 different clusters. A. log₂ fold change of clusters in WT, as well as the same genes in *MaMADS2* RNAi and *MaMADS2* antisense. B. Functional enrichment analysis of GO in the clusters (The GO functions which describes each of the functional groups are summarized in Supplementary tables S5).

Supplementary data

Fig. S1: Alignment of *MaMADS1* (EU869307) and *MaMADS2* (EU869306) partial sequences of the K C and 3'UTR regions. (Numbers of base pairs start from the beginning of the K region). Segments marked in color were used for constructing of the three types of the vectors used for banana transformation described in Table 1. Vertical line indicates the border between the K and the C regions. In yellow sequence used for *MaMADS1* pK+C RNAi (position 333-641 nucleotides of EU869307) and blue- *MaMADS2* C+3'UTR RNAi and antisense (position 520-823 of EU869306). The letters pK denotes a partial sequence of the K region.

Fig. S2: Constructs description and the verification of inserts in the transgenic lines. Schematic presentation of pHELLSGATE and pBIN vectors are depicted in A and B, respectively. Schemes in A (reactions a and b) describe the anticipated PCR products from inserts made in pHELLSGATE vectors, while in B (reaction c) describes that made in pBIN vector. The primers yielding these products are listed in Supplementary Table, S2. Letters a-c in A and B correspond to the reaction presented in C. Numbers on top of lanes in C are of plants used for characterization of ripening parameters and for gene expression analysis.

Fig. S3: Ripening parameter of control and repressed *MaMADS1* and *MaMADS2* banana fruits following ethylene treatment. Control and repressed *MaMADS1* and *MaMADS2* banana fruit were treated with ethylene (10µl/L for A or 1µl/L for B and C) immediately after harvest for one day and firmness or color was recorded in treated fruit 2 and 5 days after exposure.

Fig. S4: Up and down regulated genes between breaker to harvest in control and *MaMADS2* repressed lines. Fruits of control and transgenic RNAi-*MaMADS2* (#32) and antisense *MaMADS2* (#44) were collected at similar physiological stage on the October 2011. A. Number of genes that were up or down -

regulated by at least five times in the pulp of the different fruits. B. Number of genes that were up or down-regulated by at least five times in the peel of the different fruits.

Table 1: Description of banana transgenic lines.

Description	Beginning of hardening	Transfer to field	Harvest	PCR positive plants	Plants marks
<i>MaMADS1</i> pK + pC RNAi	16/10/2007	1/4/2008	5/5/2009	29**	4-19, 4-20 (2-8, 2-18, 2-21)***
<i>MaMADS2</i> +3'UTR RNAi ^C	6/9/2007	1/4/2008	2/6/2009	19	3-21, 3-23, 3-24
<i>MaMADS2</i> +3'UTR antisense* ^C	6/9/2007	1/4/2008	2/6/2009	2	3-45
	6/9/2007	1/4/2008	2/6/2009	16	3-36, 3-37, 3-40

* Two independent transformation were performed for this construct.

**3 plants died following their transfer to the field.

*** In parenthesis are plants which exhibit chimera as described in Fig. 4.

Letters indicate the molecule sections used for transformation as described in Supplementary, Fig. S1: C-full length of the C region; pK-partial length of the K region; 3'UTR-untranslated region at the 3' end.

References

- Alexander, L., Grierson, D.**, 2002. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *J Exp Bot* 53, 2039-2055.
- Boss, P.K., Sensi, E., Hua, C., Davies, C., Thomas, M.R.**, 2002. Cloning and characterisation of grapevine (*Vitis vinifera* L.) MADSbox genes expressed during inflorescence and berry development. *Plant Science* 162, 887-895.
- Choudhury, R.S., Roy, S., Nag, A., Singh, S.K., Sengupta, D.N.**, 2012. Characterization of an AGAMOUS-like MADS Box Protein, a Probable Constituent of Flowering and Fruit Ripening Regulatory System in Banana. *PLoS ONE* 7, e44361.
- Chung, M.-Y., Vrebalov, J., Alba, R., Lee, J., McQuinn, R., Chung, J.-D., Klein, P., Giovannoni, J.**, 2010. A tomato (*Solanum lycopersicum*) APETALA2/ERF gene, SlAP2a, is a negative regulator of fruit ripening. *Plant Journal* 64, 936-947.
- Dadzie, B.K., Orchard, J.E.**, 1997. Routine postharvest screening of banana/plantain hybrids: criteria and methods. INIBAP Technical Guidelines, Montpellier, France, p. pp75.
- Elitzur, T., Vrebalov, J., Giovannoni, J.J., Goldschmidt, E.E., Friedman, H.**, 2010. The regulation of MADS-box genes expression during ripening of banana and their regulatory interaction with ethylene. *J Exp Bot* 61, 1523-1535.
- Fujisawa, M., Shima, Y., Nakagawa, H., Kitagawa, M., Kimbara, J., Nakano, T., Kasumi, T., Ito, Y.**, 2014. Transcriptional Regulation of Fruit Ripening by Tomato FRUITFULL Homologs and Associated MADS Box Proteins. *The Plant Cell Online*.
- Giovannoni, J.J.**, 2001. Molecular biology of fruit maturation and ripening. *Ann Rev Plant Physiol Plant Mol Biol* 52, 725-749.
- Giovannoni, J.J.**, 2004. Genetic regulation of fruit development and ripening. *Plant Cell* 16, 170-180.
- Giovannoni, J.J.**, 2007. Fruit ripening mutants yield insights into ripening control. *Curr Opin Plant Biol* 10, 283-289.
- Grierson, D.**, 2013. Ethylene and the control of fruit ripening, in: Seymour, G., Giovannoni, J.J., Tucker, G., Poole, M. (Eds.), *Molecular Biology and Biochemistry of Fruit Ripening* Wiley, Boston.
- Itkin, M., Seybold, H., Breitel, D., Rogachev, I., Meir, S., Aharoni, A.**, 2009. TOMATO AGAMOUS-LIKE 1 is a component of the fruit ripening regulatory network. *The Plant Journal* 60, 1081-1095.
- Ito, Y., Kitagawa, M., Ihashi, N., Yabe, K., Kimbara, J., Yasuda, J., Ito, H., Inakuma, T., Hiroi, S., Kasumi, T.**, 2008. DNA-binding specificity, transcriptional activation potential, and the rin mutation effect for the tomato fruit-ripening regulator RIN. *Plant J* 55, 212-223.
- Karlova, R., Rosin, F.M., Busscher-Lange, J., Parapunova, V., Do, P.T., Fernie, A.R., Fraser, P.D., Baxter, C., Angenent, G.C., de Maagd, R.A.**, 2011. Transcriptome and Metabolite Profiling Show That APETALA2a Is a Major Regulator of Tomato Fruit Ripening. *Plant Cell* 23, 923-941.
- Kitagawa, M., Ito, H., Shiina, T., Nakamura, N., Inakuma, T., Kasumi, T., Ishiguro, Y., Yabe, K., Ito, Y.**, 2005. Characterization of tomato fruit ripening and analysis of gene expression in F1 hybrids of the ripening inhibitor (*rin*) mutant. *Physiol Plant* 123, 331-338.
- Klee, H.J., Giovannoni, J.J.**, 2011. Genetics and Control of Tomato Fruit Ripening and Quality Attributes. *Annual Review of Genetics* 45, 41-59.
- Lee, J.M., Joung, J.-G., McQuinn, R., Chung, M.-Y., Fei, Z., Tieman, D., Klee, H., Giovannoni, J.**, 2012. Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SlERF6 plays an important role in ripening and carotenoid accumulation. *The Plant Journal* 70, 191-204.

- Lin, Z., Arciga-Reyes, L., Zhong, S., Alexander, L., Hackett, R., Wilson, I., Grierson, D., 2008. SITPR1, a tomato tetratricopeptide repeat protein, interacts with the ethylene receptors NR and LeETR1, modulating ethylene and auxin responses and development. *J Exp Bot* 59, 4271–4287.
- Lincoln, J.E., Fischer, R.L., 1988. Regulation of gene expression by ethylene in wild-type and rin tomato (*Lycopersicon esculentum*) fruit. *Plant Physiol* 88, 370-374.
- Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J., Seymour, G.B., 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat Genet* 38, 948-952.
- Martel, C., Vrebalov, J., Tafelmeyer, P., Giovannoni, J.J., 2011. The tomato MADS-Box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. *Plant Physiology* 157, 1568-1579.
- Nguyen, C.V., Vrebalov, J.T., Gapper, N.E., Zheng, Y., Zhong, S., Fei, Z., Giovannoni, J.J., 2014. Tomato GOLDEN2-LIKE Transcription Factors Reveal Molecular Gradients That Function during Fruit Development and Ripening. *The Plant Cell Online* 26, 585-601.
- Oliveros, J., 2007. VENNY. An interactive tool for comparing lists with Venn Diagrams. BioinfoGP, CNB-CSIC.
- Pirrello, J., Regad, F., Latche, A., Pech, J.-C., Bouzayen, M., 2009. Regulation of tomato fruit ripening. *CAB Reviews* 4, 051.
- Powell, A.L.T., Nguyen, C.V., Hill, T., Cheng, K.L., Figueroa-Balderas, R., Aktas, H., Ashrafi, H., Pons, C., Fernández-Muñoz, R., Vicente, A., Lopez-Baltazar, J., Barry, C.S., Liu, Y., Chetelat, R., Granell, A., Van Deynze, A., Giovannoni, J.J., Bennett, A.B., 2012. Uniform ripening Encodes a Golden 2-like Transcription Factor Regulating Tomato Fruit Chloroplast Development. *Science* 336, 1711-1715.
- Rigola, D., Pe, M.E., Fabrizio, C., Me, G., Sari Gorla, M., 1998. CaMADS1, a MADS box gene expressed in the carpel of hazelnut. *Plant Mol Biol* 38, 1147-1160.
- Schoofs, H., 1997. The origin of embryogenic cells in Musa., Leuven, Belgium.
- Seymour, G., Poole, M., Manning, K., King, J.G., 2008. Genetics and epigenetics of fruit development and ripening. *Curr Opin Plant Biol* 11, 58-63.
- Seymour, G.B., Chapman, N.H., Chew, B.L., Rose, J.K.C., 2013. Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnology Journal* 11, 269-278.
- Seymour, G.B., Ryder, C.D., Cevik, V., Hammond, J.P., Popovich, A., King, G.J., Vrebalov, J., Giovannoni, J.J., Manning, K., 2011. A SEPALLATA gene is involved in the development and ripening of strawberry (*Fragaria×ananassa* Duch.) fruit, a non-climacteric tissue. *Journal of Experimental Botany* 62, 1179-1188.
- Sharan, R., Maron-Katz, A., Shamir, R., 2003. CLICK and EXPANDER: a system for clustering and visualizing gene expression data. *Bioinformatics* 19, 1787-1799.
- Sung, S.K., Moon, Y.H., Chung, J.E., Lee, S.Y., Park, H.G., An, G., 2001. Characterization of MADS box genes from hot pepper. *Mol Cells* 11, 352-359.
- Tadiello, A., Pavanello, A., Zanin, D., Caporali, E., Colombo, L., Rotino, G.L., Trainotti, L., Casadoro, G., 2009. A PLENA-like gene of peach is involved in carpel formation and subsequent transformation into a fleshy fruit. *J Exp Bot* 60, 651–661.
- Vrebalov, J., Matas Arroyo, A.J., McQuinn, R., Rose, J., Chung, M.-Y., Poole, M., Seymour, G., Irish, V., Giovannoni, J.J., 2009. Fleshy fruit expansion and ripening are regulated by the tomato MADS-box transcription factor TAGL1. *Plant Cell* 21, 3041-3062.
- Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Schuch, W., Giovannoni, J., 2002. A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (*rin*) locus. *Science* 296, 343-346.
- Yao, J., Kvarnheden, A., Morris, B., 1999. Seven MADS-box genes in apple are expressed in different parts of the fruit. *J Am Soc Hort Sci* 124, 8-13.

Zhong, S., Fei, Z., Chen, Y.-R., Zheng, Y., Huang, M., Vrebalov, J., McQuinn, R., Gapper, N., Liu, B., Xiang, J., Shao, Y., Giovannoni, J.J., 2013. Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nat Biotech* 31, 154-159.

Zhu, M., Chen, G., Zhou, S., Tu, Y., Wang, Y., Dong, T., Hu, Z., 2014. A New Tomato NAC (NAM/ATAF1/2/CUC2) Transcription Factor, SINAC4, Functions as a Positive Regulator of Fruit Ripening and Carotenoid Accumulation. *Plant and Cell Physiology* 55, 119-135.