Author's response to reviews

Title: Role for the banana AGAMOUS-like gene MaMADS7 in regulation of fruit ripening and quality

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Author's response to reviews:

Dear Dr. Robert Schaffer

Thanks very much for you and the reviewers' valuable comments. We have revised the manuscript carefully according to the reviewers' suggestions as marked in red in the revised manuscript and responses are made now according to the reviewers' comments point-by-point below:

1. Please include a 'Competing interests' section between the Conclusions and Authors' contributions.

Response: 'Competing interests' section has been included between the Conclusions and Authors' contributions on Page 18 Line 6-7.

Responses to Reviewer 1's comments:

The author describe the cloning and characterisation of a MADS box transcription factor from banana. In my opinion the manuscript suffers on a number of points:

1. the gene in question MsMADS7 was identified through RACE PCR. Nowhere in the manuscript is there mention of the banana genome or the full extent of the MADS gene family, this needs to be added to provide a more comprehensive perspective.

Response: The 705 bp full-length MaMADS7 cDNA was identified through RACE PCR. 5’ and 3’ RACE were conducted using the double-stranded cDNA from banana fruit at 2 DAH as a template according to the method of Liu et al. (2009) (Liu J, Xu B, Hu L, Li M, Su W, Wu J, Yang J, Jin Z: Involvement of a banana MADS-box transcription factor gene in ethylene-induced fruit ripening. Plant Cell...
Rep 2009, 28:103-111.), which was added on Page14 Line8-12. In addition, to provide the full extent of the MADS gene family is really more comprehensive, however, this report focused on the role of MaMADS7, and other MADS-box genes of banana have been mentioned in Fig.1 and the manuscript in Page 5 Line9-10 and Page 9 Line 12-22.

2. The expression analysis suffers because only one MADS gene is considered. At the very least expression of the other MADS genes in the same clad as MADS7 needs to be included to provide a context for the relative expression levels of MADS7.

Response: Thank you very much for pointing out this problem! The expression pattern of other MADS genes in the same clad as MaMADS7 were MaAG (Choudhury et al., 2012), MuMADS1 (Liu et al., 2009) and MaMADS5 (Elitzur et al., 2010), which were mentioned on Page 9 Line24-26. They are highly expressed in fruits. Those published data came from different researchers ensure that the expression characteristics of AG-like MADS-box genes are convincing.

3. Fig 1B needs to be derived from alienable sequence only and bootstrap confidence added.

Response: All these sequences in Figure 1B were derived from alienable sequence and bootstrap confidence was added. Moreover, the sequence of MaMADS7 has also been deposited in Genbank with the Accession number: KJ433373.

4. The GUS trans-activation assays seem very preliminary. GUS level alone are not a good measures of promoter trans-activation; some internal control (such as the commonly used LUC: REN ratio) are needed to make this data of any value.

Response: Thank you very much for your suggestion! GUS level alone here is not a good measure for promoter trans-activation and LUC: REN ratio was really more scientific. Because we mastered limited experimental technique, it is difficult for us to study the methods of LUC: REN ratio to measure promoter trans-activation during the short period. We will take your valuable suggestion by using LUC: REN ratio to measure promoter trans-activation further. In the present study GUS level is to provide a circumstantial evidence supports the Y1H result.

5. It is unclear where the GFP expression P7 L12 comes from.

Response: MaMADS7 was subcloned between the cauliflower mosaic virus (CaMV) 35S promoter and the green fluorescent protein (GFP) reporter gene in the pCAMBIA1302 plant transformation vector. Therefore we generated the plant transformation vector 35S: MaMADS7: GFP (pCAMBIA1302- MaMADS7-GFP) (Page14, Line32). The 35S: MaMADS7: GFP (pCAMBIA1302- MaMADS7-GFP) vector was not only used to investigate the subcellular localization of MaMADS7 in N. benthamiana leaves and but also to transform tomato (Page17, Line2). Thus, transgenic tomato lines can express MaMADS7-GFP fusions. Then, the IMG, MG and RM fruit freehand sections of transgenic tomato line L12 were inspected by confocal laser scanning microscopy to detect the expression of MaMADS7-GFP fusions in transgenic tomato. Although the images were not
clear, it was observed that MaMADS7-GFP fusions were expressed in transgenic tomato and mainly located in the nucleus (Additional Figure1). Additionally, from the figure 2B, we can also observe that the MaMADS7–GFP fusion protein accumulated in the nucleus.

6. MADS7 expression if proposed to have a time component but Fig 5A does not demonstrate accelerated ripening as suggested in the text.

Response: In the original manuscript Figure 5A, we want to display the earlier ripening of transgenic tomato than WT. When transgenic tomato reached IMG, MG, BR, RM stages, the WT did not achieve these ripening stages. However, we did not clearly describe these results. Therefore, to clearly demonstrate this point, we included the statistical data into the revised manuscript. The number of days needed for the fruit to reach the BR and RM stages after pollination were analyzed as shown on Page 7 Line 24-27(Fig. 5B in the revised manuscript).

After pollination, the WT, L12, L13, and L14 took 51, 47, 48, and 46 days to reach the BR stage and 71, 67, 68, and 66 days to reach the RM stage, respectively. These results indicated that fruit from L12, L13, and L14 plants reached the BR and RM stages 4, 3, and 5 days earlier than fruit from the WT, respectively. (Fig. 5A, B). Thus, ectopic expression of MaMADS7 in tomatoes resulted in short development stages and rapid ripening.

Responses to Reviewer 2's comments:

This body of work advances our understanding of ripening in banana and provides some insight into the role that the transcription factor MADS7 may play in the regulation of ACO and therefore ripening. In general, the paper was easy to read (well-written). The following comments though should be addressed:

1. Major Compulsory Revisions

1.1 The relationship between the regulation of ACO1 by MADS7 and the downstream genes such as PSY1 has not been explicitly discussed (in the discussion) but rather the assumption is that the reader will automatically make the link. They will if they are an expert in the field but if they are not entirely familiar with ethylene pathways and ripening they may not. I therefore suggest that some more time is spent on this in the discussion. This leads me to the next comment and a very real concern I have about overstating what the results mean.

Response: This is a good suggestion. The relationship between the regulation of ACO1 by MADS7 and the downstream genes such as PSY1, is now discussed in the Discussion section on Page 11 Lines 13-15 in red color. Moreover, the result has also been changed to right state what the results mean as shown on Page2 Line 12-13, Page5 Line32, Page6 Line22-23, Page9 Line30, Page11 Line34.

1.2 The abstract states that 'MADS7 interacts with the ACO gene promoter to regulate ethylene biosynthesis' - while this paper clearly demonstrates that MADS7 and the ACO gene promoter interact, I do not believe that the evidence (of overexpression increasing ethylene production necessarily is conclusive). Either the authors be more careful in this interpretation or they provide evidence in the form of promoter deletion analysis especially with potential binding
elements removed. Alternatively knockdown mutants may be preferable but I realise this may be difficult (as I realise that banana is not the easiest transformation system).

Response: Thank you very much for your valuable suggestion! The description of 'MADS7 interacts with the ACO gene promoter to regulate ethylene biosynthesis' was not appropriate. Although MADS7 can interact with the MaACO1 gene promoter in banana, there are no sufficient evidences to determine their interaction regulating ethylene biosynthesis. MADS7 can interact with the MaACO1 gene promoter in banana, implying the function of MADS7 in ethylene biosynthesis. Additionally, ectopic expression of MaMADS7 in tomato resulted in short development stages, rapid ripening and increased ethylene evolution and expression of MaACO1. These results allow us to conclude that MaMADS7 expression could promote ethylene biosynthesis. Therefore, the description of 'MADS7 interacts with the ACO gene promoter to regulate ethylene biosynthesis' was carefully revised according to your suggestions on Page2 Line 12-13, Page5 Line32, Page6 Line22-23, Page9 Line30, Page 10 Line 1-26, Page11 Line34.

1.3 The use of statistical significance is essential to any scientific work and this is not evident in this manuscript (eg ANOVAs for the qPCR data?). It may affect the interpretation of the data. In particular:

Response: Statistical significances to any scientific work in this manuscript have been added as shown in the revised manuscript.

- Line 7 Page 6 - How is the ethylene production by the 1-MCP treatment relatively high? Relative to what? It is actually very low compared to the other treatments - this needs to be interpreted correctly.

Response: Thank you very much for pointing out this problem!

The ethylene production by the 1-MCP treatment was relatively high at 0 DAH compared to other time points with the same treatment, which has been adjusted in the revised manuscript as shown on Line 7 Page 6. In addition, these results were further explained to make reviewers' and readers' clearly understand the meaning of the results. A MaMADS7 expression peak appeared at the initiating stage of endogenous ethylene biosynthesis both in naturally ripened fruit or fruits treated with exogenous ethylene (Fig. 3A, B, D and E). Moreover, MaMADS7 expression was greatly induced by exogenous ethylene and suppressed by 1-MCP during post-harvest banana fruit ripening (Fig. 3E, F). These results suggested that MaMADS7 responds to ethylene (Page6 Line12-23).

- Line 10 Page 6 states that expression of MADS7 was relatively high but again, in relation to what and the comment on Line 12 in next sentence that there is a second small peak should have statistics to support it - the standard error bars appear to overlap so day 4 and 8 may in fact be no different. The statistical analysis should be done and LSDs used to determine whether in fact there are differences.

Response: The expression level of MaMADS7 in naturally ripened fruit was relatively high at the initiating stage of endogenous ethylene biosynthesis.
compared to other time points with the same treatment, which has been adjusted in the revised manuscript as shown in red on Page 6 Line 12. The statistical analysis was performed and LSDs were used to determine whether there were significant differences. Data are means ± SE of n = 3 biological replicates. Means denoted by the same letter do not significantly differ at P < 0.05 as determined by LSDs multiple range test.

1.4 3 replicates and only one experiment seems rather minimal for the data shown in Figure 3 - However, others have already shown this type of ethylene production - the authors should mention they are confirming those published data or else ensure they have enough replication to show the data is convincing.

Response: Three replicates and only one experiment seem really minimal for the data shown in Figure 3. We focus on postharvest banana ripening study for long time. The measurement of ethylene production is an important basis for our studies. Therefore, we also pay close attention to the ethylene production in postharvest banana ripening. In our previous studies, almost each series of banana fruits were subjected to ethylene production measurement. Thus, the measurement system is very stable in our laboratory. The trends of ethylene evolution in naturally ripened, ethylene treated and 1-MCP treated banana are consistent with previous studies (Liu et al. 2013; Jia et al. 2012; Jia et al. 2013).

Liu et al. (2013) Function of a citrate synthase gene (MaGCS) during postharvest banana fruit ripening. Postharvest Biology and Technology, 84: 43-50


1.4 Page 5 Line 20 states that MADS7 was expressed in the style/stigmas of the female flower - the Figure 2A does not show this.

Response: From the Figure 2A, Because of the extremely low expression level, it is difficult to observe that MADS7 was expressed in the style/stigmas of the female flower. In the revised manuscript, the expression of MADS7 was appropriately revised to make it easily understand (Page5 Line21-22).

1.5 Line 15 Page 6 - The comment about Figure 3F suggests the authors are talking about all timepoints but it is not clear (as only after Day 0 is the expression suppressed).

Response: The comment about Figure 3F has been changed to “MaMADS7 expression in 1-MCP- treated fruit was greatly suppressed after 0 DAH” on Page 6 Line 17.

2. Minor Essential Revisions

2.1 Line 10 Page 3 - biosynthesis has been spelt incorrectly and needs Correcting

Response: Thank you very much for pointing out this problem!
The word ‘biosynthesis’ has been spelled correctly in the revised version.

2.2 Line 7 Page 3 - please make sure the 2 in carbon dioxide is subscripted.
Response: Corrected.

2.3 Line 4 Page 4 - ‘The suite of’ (Suite should be singular)
Response: I corrected it.

2.4 Line 17 Page 5 - 'belonged' should be 'belongs'
Response: Corrected.

2.5 Line 17 Page 7 - The sentence about L12, L13 and L14 plants is hard to follow - perhaps change to: ‘Fruit from L12, L13 and L14 plants reached the RM stage 4, 3 and 5 days earlier than fruit from the WT, respectively’
Response: I corrected it following your suggestion.

2.6 Page 10 heading - remove 'in a broad scope' - not appropriate English
Response: Corrected.

2.7 Figure 2 Legend - Representative of ? samples and experiments? (again -links to demonstrating all statistical analysis)
Response: Thank you for your suggestion! Experiments were performed in triplicate, which was added in the revised manuscript as shown in red on Page 24 Line 32.

2.8 Figure 3 legend - ppm ethylene? LSDs? What was expression relative to? At what temperature were they stored?
Response: Thank you very much for pointing out these problems! The contents of ethylene and 1-MCP and the stored temperature have been added. The expression was relative to Maactin1, which has also been added in the Figure 3 legend.

2.9 Figure 6 - suddenly has SD and yet earlier SE were used - be consistent. LSDs? Means of how many replicates?
Response: Thank you very much for pointing out these problems! The SD in Figure 6 has been changed to SE, which is consistent with Figure 3 and 5.

2.10 Just a general note about the tidiness of the graphs and the placement of the labels A, B, C etc..... It is usual to have them on the upper left hand side of each sub-figure - things should also be aligned
Response: I have corrected as you suggested.

Responses to Reviewer 3's comments:

This manuscript outlines the study of a MADbox gene of the agamous clade from the important fruit species banana, the authors propose to have a role in regulating fruit ripening. This is a timely translational biology research study following the characterization of the tomato agamous-like1 gene during ripening. However, in its current state, I cannot recommend this manuscript be accepted for publication.
1) The main concern I have relates to the developmental age of the fruit used in this study. MaMAD7 was ectopically expressed, 3 transgenic lines were analyzed, and compared to WT, but there is no mention as to how their developmental age of these fruit was assessed. There is no data supporting early ripening statement….3-4 days early. Figure 5 is referenced, which shows redder fruit but methods make no mention of tagging fruit or how they came to this conclusion. The only way is tagging pollinated flowers, and comparing the days until ripening starts. In addition, fruit at the same developmental stage can be tagged and staged at the Breaker stage (the first visual sign of the onset of ripening), and the time course assessed from there.

Response: Thank you very much for your valuable suggestions! The developmental age of these fruits was assessed by tagging pollinated flowers, which was added in the Method section on Page 12 Line30 as shown in the revised manuscript in red. 'The early ripening statement….3-4 days early' was supported by the calculated number of days needed to reach BR and RM stages after pollination as shown in the revised manuscript (Fig. 5B).

Again, the most red stage (RM) of the WT, is not in fact what I am accustomed to as a full red ripe fruit. Red ripe lacks any of the orange pigment to the eye, as is still observable in this figure for WT red fruit. Further, the Breaker stage fruit should all look the same if they are all at the same stage of development. The transgenics are certainly more advanced in their ripening state, but certainly not 3-4 days more advanced as proposed by the authors. These inconsistencies should be addressed, and the exact manner in how they staged these fruit should be elaborated on. Something that is not staged properly here, then calls into question all the gene expression and metabolite data. In addition, a stage in between Br and RM would be useful in assessing changes in ripening state.

Response: In the original manuscript Figure 5A, we want to display the earlier ripening of transgenic tomato than WT. When transgenic tomato reached IMG, MG, BR, RM stages, the WT did not achieve these ripening stages. However, we did not clearly descript these results. Therefore, to clearly demonstrate this point, we included the statistical data into the revised manuscript. The number of days needed for the fruit to reach the BR and RM stages after pollination were analyzed as shown on Page 7 Line 24-27 (Fig. 5B in the revised manuscript). After pollination, the WT, L12, L13, and L14 took 51, 47, 48, and 46 days to reach the BR stage and 71, 67, 68, and 66 days to reach the RM stage, respectively. These results indicated that fruit from L12, L13, and L14 plants reached the BR and RM stages 4, 3, and 5 days earlier than fruit from the WT, respectively. (Fig. 5A, B). Thus, ectopic expression of MaMADS7 in tomatoes resulted in short development stages and rapid ripening. Fruits of different developmental stages were assessed by tagging pollinated flowers according to reference [32]: immature green (IMG) [17 days after pollination (DAP)], mature green (MG), breaker fruit (BR) and red mature (RM) in normal and transgenic plants were harvested for further physiological analyses and genes expression assessment (Page12 Line30-33).
2) The statement that MaMADS7 responds to ethylene is consistent with their results. However their statement that it controls or influences ethylene is not substantiated at all. As such the heading on bottom of page 5 should be changed. The text in that paragraph should also be changed. The text in the next paragraph (it binds to the ACO promoter) would provide justification for this statement. Discussion on page 9 again does not support this......the promoter binding does but the correlated expression with ethylene likely could be said for hundreds if not thousands of genes. It is a small text distinction but has major implications.

Response: The description of 'MADS7 interacts with the ACO gene promoter to regulate ethylene biosynthesis' was not appropriate. Although MADS7 can interact with the MaACO1 gene promoter in banana, there are no sufficient evidences to determine their interaction regulating ethylene biosynthesis. MADS7 can interact with the MaACO1 gene promoter in banana, implying the function of MADS7 in ethylene biosynthesis. Additionally, ectopic expression of MaMADS7 in tomatoes resulted in short development stages, rapid ripening and increased ethylene evolution and expression of MaACO1. These results allow us to conclude that MaMADS7 expression could promote ethylene biosynthesis. Therefore, the description of 'MADS7 regulate ethylene biosynthesis' was carefully discussed on Page 10 Line 1-26)

Minor

3) Ethylene release/production should be changed to ethylene evolution (eg Fig 3 & 5C)

Response: Ethylene release/production has been changed to ethylene evolution in Fig 3 and 5C and the whole text of the revised manuscript.

4) The broken y-axis for the gene expression data in figure 6 is confusing, my suggestion is to just leave it out. The small differences in expression the authors are trying to show in these graphs are probably less relevant than the large changes observed.

Response: The broken y-axis for the gene expression data in Figure 6 has been left out as shown in the revised manuscript.

5) minor grammatical and editorial corrections should be made in the text.

Response: The editorial corrections have been made in the text.

6) std error was used in figure 3 & 5, why the change to std dev for fig 6? Consistency I think is best policy here.

Response: The std dev for Figure 6 has been changed to std error for consistency with Figures 3 and 5.

7) In figures 3 and 6, what is the expression normalized/relative to?

Response: In Figure 3, the expression is normalized to Maactin1. In Figure 6, the expression is normalized to 18S as shown in Additional file 2 and Page 13 Line 31-32.
We hope that these revisions are satisfactory and that the revised version will be acceptable for publication. At last, we are impressed by your professional and religious attitude. Thank you very much for your work concerning our paper.

Best Wishes!

Sincerely yours,

Zhiqiang Jin