



Gene regulatory network of carpel number variation in cucumber

Gen Che, Ran Gu, Jianyu Zhao, Xiaofeng Liu, Xiaofei Song, Hailing Zi, Zhihua Cheng, Junjun Shen, Zhongyi Wang, Renyi Liu, Liying Yan, Yiqun Weng and Xiaolan Zhang
DOI: 10.1242/dev.184788

Editor: Yka Helariutta

Review timeline

Original submission:	17 September 2019
Editorial decision:	4 November 2019
First revision received:	24 January 2020
Accepted:	26 February 2020

Original submission

First decision letter

MS ID#: DEVELOP/2019/184788

MS TITLE: Gene regulatory network of carpel number variation in cucumber

AUTHORS: Gen Che, Ran Gu, Jianyu Zhao, Xiaofeng Liu, Xiaofei Song, Hailing Zi, Zhihua Cheng, Junjun Shen, Zhongyi Wang, Renyi Liu, Liying Yan, Yiqun Weng, and Xiaolan Zhang

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to [BenchPress](#) and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but Reviewer 2 still has some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Che et al. describes the functional analysis of the CLV3-WUS module in cucumber. The results presented show that this module affects carpel number. Increased WUS expression (OX) gives more carpels and reduced CLV3 expression (RNAi) results in less carpels, based on generated transgenic lines in cucumber. The GFC line produces fruits with 5 carpels (commercial lines have fruits with 3 carpels), a correlation is presented for WUS and CLV3

expression. All phenotypes are nicely documented, expression for the genes is presented by in situ hybridization in the different lines and by qRT-PCR. Furthermore, a previously published overexpression line of FUL (OX) is used that shows reduced CLV3 expression and increased WUS expression; this line presents more floral organs, including increased carpel number. The authors show that FUL regulates directly WUS, shown by Y1H, transactivation assay in plants and by ChIP. The study is followed by an RNA-seq study comparing two lines: 32X versus GFC. Enriched in transcription factors, which the authors hypothesize can be linked to hormone regulation. Hormone levels are measured, and IAA and ABA are both reduced in the GFC line. Immunolocalization is performed with anti-IAA, showing there is a slight reduction in signal in the GFC line. The final part of the study is the identification of the ARF14 protein as an interactor of the WUS protein, based on Y2H and BiFC. A model is presented at the end. The manuscript presents data of high quality, nice figures, all well presented in a nice story. English is overall good.

Comments for the author

1. Please add additional information on the RNA-seq study in the M&M. Number of samples; seems to be in triplicate seeing the heatmap, please confirm. A suppl. Table could be added on the number of reads obtained, mapped reads, etc.
2. Based on the RNA-seq experiment, the jump goes from transcription factors directly to hormones. Did the authors found ARFs?, and what happened with CLV3, WUS, and FUL? Maybe a few lines could be mentioned.
3. Hormone measurements, the authors show that IAA and ABA are less present in the GFC line, and focus on IAA. But what about ABA? Can the authors discuss a bit if there is a relation between ABA and carpel number?
4. The authors mentioned that they also measured cytokinin, but do not comment on the outcome. Was it really measured? Please comment (or correct).
5. Maybe I missed it, but did the authors check ARF14 expression in the GFC line?
6. Could the authors speculate if WUS function is dependent on ARF14? What is the role of the dimer WUS-ARF14?
7. A technical remark, it would be good to show the negative controls for the BiFC experiment.
8. It would be good to mention in the M&M which antibody against IAA is used, and not only a reference.
9. Perhaps the authors missed the work on CLV3-WUS in tomato on carpel number by Rodriguez-Leal et al (2017) Cell, would be nice to cite this work as well.

Reviewer 2

Advance summary and potential significance to field

In the submitted manuscript, Che et al., explored a regulatory loop that specifies carpel development in cucumber. Specifically, they performed the phylogenetic analysis of CsCLV3 and CsWUS and determined the expression patterns of these two genes through the RNA in situ hybridization. They characterized the CsCLV3 RNAi and CsWUS overexpression transgenic plants. In addition, they examined the new role of CsFUL1 in control of carpel number and tested the direct activation of CsWUS by CsFUL1 both in yeast and in vivo. They also examined the physical interaction between CsWUS and CsARF14, which may relate to the activity of Auxin in control of carpel development. The findings in the manuscript uncover the function of several key genes in regulating an important developmental process, which are of interest.

Comments for the author

Major concerns:

It has been reported that CsCLV3 is the key player in control of the carpel number in cucumber (Integrated analysis in bi-parental and natural populations reveals CsCLAVATA3 (CsCLV3) underlying carpel number variations in cucumber. Theoretical and Applied Genetics May 2016, Volume 129, Issue 5, pp 1007-1022), and it seems that the CsCLV3 work here does not provide new insight on this system.

The authors proposed a regulatory loop between CsCLV3 and CsWUS in control of carpel number in cucumber. However, more experiments with careful analyses are needed to test the proposed model here. 1) CsCLV3 RNAi does not lead to misregulation of CsWUS (Fig 2O), suggesting CsCLV3 signaling does not regulate CsWUS. Since the phenotype of CsCLV3 RNAi is obvious (Fig 2D-M, P), the authors' explanation "which may due to the reduction of CsCLV3 in RNAi lines is not enough to repress CsWUS expression, and/or other genes function redundantly with CsCLV3 to modulate CsWUS expression in the female flower of cucumber" is not very convincing. 2) It is interesting that overexpression of CsWUS leads to the reduction of CsCLV3 (Fig 3I). Does CsWUS directly repress CsCLV3? Following authors' discussions, does CsWUS regulate CsCLV3 expression in a concentration dependent way? If CsWUS negatively regulates CsCLV3, directly or indirectly, how to explain the completely overlapping expression patterns of CsCLV3 and CsWUS (Fig 1)?

It's interesting and a bit surprising that CsFUL1 binds to all four DNA regions (pW1, pW2, pW3 and pW4) from the CsWUS promoter (Fig 4 L, N). Is the interaction dependent on the CArG box? In general, does CsFUL1 bind all the CArG-box containing DNA fragments? In addition, instead of showing the RNA in situ to CsCLV3 (Fig 4H), it will be more informative to perform the RNA in situ to probe the CsWUS expression pattern in the CsFUL1 OX line.

A few minor points:

To validate the specificity of the IAA immunolocalization results (Fig 5F), it will be better to include both negative and positive controls in the experiment, and quantify the signal, if possible.

On Page 15, several references reporting the activation of CLV3 by WUS and the repression of WUS by CLV3 signaling in Arabidopsis are missing (Schoof et al., The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. 2000; Brand et al., Regulation of CLV3 expression by two homeobox genes in Arabidopsis. 2002; Müller et al., Dynamic and compensatory responses of Arabidopsis shoot and floral meristems to CLV3 signaling. 2006).

First revision

Author response to reviewers' comments

Reviewer 1 Advance summary and potential significance to field

The manuscript by Che et al. describes the functional analysis of the CLV3-WUS module in cucumber. The results presented show that this module affects carpel number. Increased WUS expression (OX) gives more carpels and reduced CLV3 expression (RNAi) results in less carpels, based on generated transgenic lines in cucumber. The GFC line produces fruits with 5 carpels (commercial lines have fruits with 3 carpels), a correlation is presented for WUS and CLV3 expression. All phenotypes are nicely documented, expression for the genes is presented by in situ hybridization in the different lines and by qRT-PCR. Furthermore, a previously published overexpression line of FUL (OX) is used that shows reduced CLV3 expression and increased WUS expression; this line presents more floral organs, including increased carpel number. The authors show that FUL regulates directly WUS, shown by Y1H, transactivation assay in plants and by ChIP. The study is followed by an RNA-seq study comparing two lines: 32X versus GFC. Enriched in transcription factors, which the authors hypothesize can be linked to hormone regulation. Hormone levels are measured, and IAA and ABA are both reduced in the GFC line. Immunolocalization is performed with anti-IAA, showing there is a slight reduction in signal in the GFC line. The final part of the study is the identification of the ARF14 protein as an interactor of the WUS protein, based on Y2H and BiFC. A model is presented at the end.

The manuscript presents data of high quality, nice figures, all well presented in a nice story. English is overall good.

Reviewer 1 Comments for the author

1. Please add additional information on the RNA-seq study in the M&M. Number of samples; seems to be in triplicate seeing the heatmap, please confirm. A suppl. Table could be added on the number of reads obtained, mapped reads, etc.

Response: Yes, we have three replicates for RNA-seq. Additional information has been added as suggested, including a suppl. Table S1 with number of reads and mapped reads, and detailed description in M&M section.

2. Based on the RNA-seq experiment, the jump goes from transcription factors directly to hormones. Did the authors found ARFs? and what happened with CLV3, WUS, and FUL? Maybe a few lines could be mentioned.

Response: Yes, we found two ARFs were upregulated in 32X. Because the expression levels of CLV3 and WUS were too low (average reads of 7 and 10.5), they were excluded during data normalization of RNA-seq. However, we showed that CsCLV3 expression was significantly reduced and CsWUS expression was increased in GFC compared to 32X by qRT-PCR analyses (Fig 5C-D). In both 32X and GFC, it is homozygous CsFUL1C, not CsFUL1A. Despite the expression of CsFUL1C was significantly decreased in GFC (see Table S2), our data showed that it is CsFUL1A, not CsFUL1C, that regulates carpel number variation and fruit length in cucumber.

3. Hormone measurements, the authors show that IAA and ABA are less present in the GFC line, and focus on IAA. But what about ABA? Can the authors discuss a bit if there is a relation between ABA and carpel number?

Response: Because there are no reports about ABA in carpel number regulation in planta, we only focus on IAA in this manuscript. As suggested, we add one sentence 'Similar to the reduced auxin contents, the ABA level was less accumulated in the GFC (Fig S4), but the specific roles of ABA in cucumber carpel number variation need further investigation' in the Discussion (lines 464-466).

4. The authors mentioned that they also measured cytokinin, but do not comment on the outcome. Was it really measured? Please comment (or correct).

Response: Yes, in addition to auxin and ABA, we also measured the contents of cytokinin (ZR: trans-zeatin riboside), gibberellins (GA3), jasmonic acid (JA), and brassinosteroid (BR) in 32X and GFC, and no significant differences were observed for ZR, GA3, JA and BR (supplemental Figure 4), we add this result in line 329 in the revised manuscript.

5. Maybe I missed it, but did the authors check ARF14 expression in the GFC line?

Response: We appreciate this comment and examined ARF14 expression as suggested. Our data showed that the expression of ARF14 was significantly upregulated in GFC and CsWUS-OX plants (Fig. 5I-J), displaying a positive correlation with carpel numbers (see lines 329-341).

6. Could the authors speculate if WUS function is dependent on ARF14? What is the role of the dimer WUS-ARF14?

Response: We appreciate this insightful comment. Our data indicated that CsWUS directly interacts with CsARF14 at protein level (Fig. 5G-H). Overexpression of CsWUS led to increased number of carpel and elevated expression of CsARF14 (Fig. 3C-G, Fig. 5J). These data suggested that the expression of CsARF14 is dependent on CsWUS function. However, whether CsARF14 feeds back on CsWUS function, and what is the role of CsWUS-CsARF14 dimer need further investigation via knockout of CsARF14 in cucumber, which is time consuming and beyond the scope of this manuscript.

7. A technical remark, it would be good to show the negative controls for the BiFC experiment.

Response: Added as suggested (Fig 5H).

8. It would be good to mention in the M&M which antibody against IAA is used, and not only a reference.

Response: Added as suggested (see lines 539-543).

9. Perhaps the authors missed the work on CLV3-WUS in tomato on carpel number by Rodriguez-Leal et al (2017) Cell, would be nice to cite this work as well.

Response: Cited as suggested (see line 126 and line 165).

Reviewer 2 Advance summary and potential significance to field

In the submitted manuscript, Che et al., explored a regulatory loop that specifies carpel development in cucumber. Specifically, they performed the phylogenetic analysis of CsCLV3 and CsWUS and determined the expression patterns of these two genes through the RNA in situ hybridization. They characterized the CsCLV3 RNAi and CsWUS overexpression transgenic plants. In addition, they examined the new role of CsFUL1 in control of carpel number and tested the direct activation of CsWUS by CsFUL1 both in yeast and in vivo. They also examined the physical interaction between CsWUS and CsARF14, which may relate to the activity of Auxin in control of carpel development. The findings in the manuscript uncover the function of several key genes in regulating an important developmental process, which are of interest.

Reviewer 2 Comments for the author

Major concerns:

It has been reported that CsCLV3 is the key player in control of the carpel number in cucumber (Integrated analysis in bi-parental and natural populations reveals CsCLAVATA3 (CsCLV3) underlying carpel number variations in cucumber. Theoretical and Applied Genetics May 2016, Volume 129, Issue 5, pp 1007-1022), and it seems that the CsCLV3 work here does not provide new insight on this system.

Response: In the published paper (Theoretical and Applied Genetics May 2016, Volume 129, Issue 5, pp 1007-1022), CsCLV3 was shown to be the candidate gene for carpel number variation. However, no functional verification was performed, neither is known CsCLV3 is a positive or negative regulator for carpel number. Here we functionally showed that CsCLV3 is a negative regulator for carpel number variation in cucumber.

The authors proposed a regulatory loop between CsCLV3 and CsWUS in control of carpel number in cucumber. However, more experiments with careful analyses are needed to test the proposed model here. 1) CsCLV3 RNAi does not lead to misregulation of CsWUS (Fig 2O), suggesting CsCLV3 signaling does not regulate CsWUS. Since the phenotype of CsCLV3 RNAi is obvious (Fig 2D-M, P), the authors' explanation "which may due to the reduction of CsCLV3 in RNAi lines is not enough to repress CsWUS expression, and/or other genes function redundantly with CsCLV3 to modulate CsWUS expression in the female flower of cucumber" is not very convincing. 2) It is interesting that overexpression of CsWUS leads to the reduction of CsCLV3 (Fig 3I). Does CsWUS directly repress CsCLV3? Following authors' discussions, does CsWUS regulate CsCLV3 expression in a concentration dependent way? If CsWUS negatively regulates CsCLV3, directly or indirectly, how to explain the completely overlapping expression patterns of CsCLV3 and CsWUS (Fig 1)?

Response: We highly appreciate these insightful comments and additional experiments with careful analyses were performed as suggested.

1) Considering that carpel number is determined very early during flower development, the samples (ovary) we used for expression analyses previously were too late to detect the real change. Therefore, we performed expression analyses of CsCLV3 and CsWUS using 16-day and 20-day apex as samples. Our new data showed that CsCLV3 expression was significantly reduced, while CsWUS expression was greatly enhanced in the CsCLV3-RNAi lines (Fig 2N-O). Similarly, using 16-day and 20-day apex as samples, we found that CsWUS and CsCLV3 was significantly upregulated in the CsWUS overexpression lines (Fig 3H-I). These data are consistent with the classical negative feedback loop between WUS and CLV3.

2) Using yeast one hybrid, LUC transaction assay and ChIP-qPCR, we found that CsWUS can directly bind to the promoter of CsCLV3 to activate CsCLV3 expression (Fig 3J-N).

It's interesting and a bit surprising that CsFUL1 binds to all four DNA regions (pW1, pW2, pW3 and pW4) from the CsWUS promoter (Fig 4 L, N). Is the interaction dependent on the CARG box? In general, does CsFUL1 bind all the CARG-box containing DNA fragments? In addition, instead of showing the RNA in situ to CsCLV3 (Fig 4H), it will be more informative to perform the RNA in situ to probe the CsWUS expression pattern in the CsFUL1 OX line.

Response: We found six CARG-box located at the promoter region of CsWUS sequence, and CsFUL1A can bind to four of them. We performed in situ hybridization of CsWUS on the CsFUL1A-OX plants as suggested. Our data showed that the expression domain of CsWUS was expanded in the CsFUL1-OX plants (Fig 4H-M), consistent with positive regulation of CsWUS expression by CsFUL1A in cucumber.

A few minor points:

To validate the specificity of the IAA immunolocalization results (Fig 5F), it will be better to include both negative and positive controls in the experiment, and quantify the signal, if possible.
Response: Negative and positive controls were added as suggested (Fig 5F).

On Page 15, several references reporting the activation of CLV3 by WUS and the repression of WUS by CLV3 signaling in Arabidopsis are missing (Schoof et al., The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. 2000; Brand et al., Regulation of CLV3 expression by two homeobox genes in Arabidopsis. 2002; Müller et al., Dynamic and compensatory responses of Arabidopsis shoot and floral meristems to CLV3 signaling. 2006).

Response: Cited as suggested (see lines 395 and 425).

Second decision letter

MS ID#: DEVELOP/2019/184788

MS TITLE: Gene regulatory network of carpel number variation in cucumber

AUTHORS: Gen Che, Ran Gu, Jianyu Zhao, Xiaofeng Liu, Xiaofei Song, Hailing Zi, Zhihua Cheng, Junjun Shen, Zhongyi Wang, Renyi Liu, Liying Yan, Yiqun Weng, and Xiaolan Zhang

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

The manuscript by Che et al. describes the functional analysis of the CLV3-WUS module in cucumber. The results presented show that this module affects carpel number.

Comments for the author

The authors addressed all my comments. I do not have further comments. It is a nice story.

Best,
Stefan de Folter.

Reviewer 2

Advance summary and potential significance to field

In the revised manuscript, Che et al., have performed new experiments to uncover the cellular and molecular basis of carpel development in cucumber, focusing on the evolutionarily conserved CsCLV3-CsWUS regulatory loop and the novel regulators of this loop. The new results presented in the manuscript are interesting and convincing.

Comments for the author

With the significant improvement, the revised manuscript has addressed all my comments/concerns. I do not have any additional question or comment.