Dear Dr. Park,  
  
Your manuscript JoVE51340 'Lignin Down-regulation of Zea mays and Cell Wall Compositional Analyses' has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.   
  
Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

**Editorial comments:**  
  
1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.  
  
2) Prior to peer review, the protocol length is over our 3 page limit. Please use yellow highlighting to identify a total of 2.75 pages of protocol text (which includes headings and spaces) to identify which portions of the procedure are most important to include in the video, i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. See JoVE's instructions for authors for more clarification and remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.  
  
3) Section 1 would require extensive conceptualization in order to be filmed. If you want to include it in the video, then you will need to provide graphics to accompany that material.   
  
4) If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**Reviewers' comments:**  
  
**Reviewer #1:**   
*Manuscript Summary:*   
In the summary briefly mention the limitation of this gene disruption technique.

The limitations are mentioned in the introduction (line 101-102).  
  
*Major Concerns:*   
The lignin content in biomass is presented as g/kg of corn stover. However, when reporting the cellulose and hemicellulose content in the genetically modified maize plants (figure 2A and 2B), it is given as ng/mg of ?. It will be appropriate if the cellulose and hemicellulose content is presented as g/kg of corn stover.

The ng/mg has been converted to g/kg in Figure 4A.

Provide brief protocol about carbohydrate analysis instead of just referring to a manuscript.   
Brief protocol summary has been described in the line 399-402.

**Editorial comment:**  
[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments(use short steps, imperative tense, proper spacing, etc).]   
  
The authors claim that down regulating lignin will increase the digestibility of corn stover. However, no experimental evidence is provided. Additional data (enzyme hydrolysis and sugar conversion data) to prove that the down regulation of lignin increase the biomass digestibility is necessary.

The digestibility test has been added as in Figure 5.

Picture of the maize plants (wild type versus down regulated plants should be provided). This will help people who read this article to visualize any physical changes in the plants and their respective growth.   
The picture of the maize plants has been added to Figure 2.

**Reviewer #2:**   
*Manuscript Summary:*   
The manuscript entitled "Lignin Down-regulation of Zea mays and Cell Wall Compositional Analyses" describes the down-regulation of a lignin biosynthetic gene (CCR1) in maize using a dsRNAi approach. The authors describe a method for (i) construction of a dsRNAi construct, (ii) creation of transgenic maize plants by particle bombardment and (iii) analysis of cell wall structures in transgenic maize plants. Overall this article gives a detailed technical overview of the above mentioned methods and it will be of interest to many plant scientists working in the field of maize genetics and cell wall structure.   
  
*Minor Concerns:*  
To better reflect the focus of the manuscript, the authors might consider rephrasing the title and include keywords such as "dsRNAi", "particle bombardment" or similar in the title. This would help readers to immediately see which methods are described in the video.

The title has been changed to “Lignin Down-regulation of *Zea mays* via dsRNAi technique and Lignin Analysis”

The introduction gives background information for all methods described in the manuscript. However, it misses giving information about the SEM analysis mentioned in the manuscript. The Authors should add a brief paragraph about the SEM technique.

Brief SEM information has been added in lines 112 to 114.  
  
1.1: The authors should briefly explain how the silencing sequence for the dsRNAi approach was selected and which rules need to be followed to obtain successful silencing. A reference might be useful for viewers, who are interested in this technique.  
The reason chosen for the sequence is briefly added to the introduction. Please see the lines 120-123.

2.7, 2.9, 2.11: replace "ul" with "µl"

The units have been replaced.

3.1: Please list the composition of "10% neutral buffered formalin" in the "Table of specific material"

The compositions have been added to the “Table of specific material”

3.14: What kind of critical point dryer has been used, add to "Equipments" section

The information (Critical point dryer, Balzers CPD (Leica Microsysstems Inc, Buffalo Grove, IL, United States) has been added to “Equipments” section.

4.1: What kind of mill has been used, add to "Equipments" section

The information (Fitzpatrick JT-6 Homoloid mill; Continental Process Systems, Inc., Westmont, IL) has been added to Equipments section.

4.2: What kind of moisture analyzer has been used, add to "Equipments" section

The information (MA35 Moisture Analyzer; Sartorius) has been added to Equipments section.

4.4: Please give information about the screw-top high pressure tubes in the "Table of specific material"

The information (#8648-27 Pressure tube with #5845-47 Plug; Ace Glass, Vineland, NJ)  
has been added to the ‘Table of specific material’

Legend of figure 4: "gas Chromatography" should be "gas chromatography"

The “C” has been changed to “c”

Discussion, 4th paragraph, line 7: "chagnes" should be "changes"

The error has been fixed.

Figure 3 title: "Acid-Insuluble Lignin Contents" should be "Acid-Insoluble Lignin Contents"

The error has been fixed.

**Reviewer #3:**   
1) As the paper describes a protocol for maize transformation, the reader could be interested in the transformation efficiency and or the ratio between stable and transient transformants using this protocol?

It showed ~30% transformation efficiency. The ratio between stable and transient transformants was not measured in the original research (Lines 410-411).

2) Another interesting question is on the silencing stability in the plant and whether CCR is down over several generations? The silencing effects were consistently observed in several generations (T0-T2). Please see the lines 411-412.

Related to this question: Was transcription level of CCR checked in the different lines (based on the previous paper I know it was, but it is not mentioned here).

How many CCR genes do you have in maize, and do you target them all with the construct used?

Two CCR genes (ZmCCR1 and ZmCCR2) were characterized in maize. ZmCCR1 was targeted for gene silencing in this paper. Please see the lines 116-117.

3) It could be wise to change the title. Especially since I'm not convinced the cell wall composition analysis was performed in an accurate way (for sure, "cell wall analysis" was not performed on all samples). Why not sticking to the transformation and lignin analysis? of course, the conclusions on the carbon flux could not be made in that case, but even with the presented data I'm not convinced it is appropriate to make this statement (see later)

The title has been changed to “Lignin Down-regulation of Zea mays via dsRNAi and Lignin Analysis”

4) Related to previous remark: At the end of the introduction, the authors mentioned "Here we describe a procedure for ?. ? and for analysis of the plant cell wall components of these materials." I have some problems with the last statement, as only the analysis of lignin is explained. For hemicellulose and cellulose only a reference is given. In line with previous remark I would suggest to remove point 5 (Carbohydrate analysis), as this is only a reference. Similar reference is given in the introduction. Also hemicellulose analysis is only briefly mentioned in the introduction, but not explained in the protocol-section.

Yes, the authors agree with the reviewer’s comment, and the cell wall carbohydrate analysis part was removed from the last paragraph. Please see the lines 151-153.

5) Still on the cell wall analysis. Lignin was determined in following samples 1C4, 1C5, 1C6; cellulose measurements were done on the same and some additional samples; and hemicellulose still on a different subset. As a result for line 1B3 and 1B6 we only have cellulose data, and for 1B4 we have no lignin data. This makes it dangerous to make strong conclusions. For example, the conclusion that there is a carbon flow from lignin too cellulose is based on one of the seven ccr samples. And although we do have lignin and cellulose data for 1C4 and 1C5, we don't see this "carbon flow" from lignin towards cellulose. Maybe the authors put their statement a little too strong.

Yes, the authors agree with the comments. There was no direct evidence of shifting of carbon flow from lignin to cellulose. Thus the conclusion sentence in the long abstract has been changed. Please see lines 58-59.

6) For the protocol: specify the characteristics of the rupture disk (which pressure), and what distance was used?

The pressure and distance were added in the transformation protocol. Please see lines 242and 249.

7) The protocol jumps from 4.13 -> 4.18  
The numbering has been fixed.

8) In the listed formula, it seems like the authors multiply by 1, which is most likely not the case. Brackets should be added to clarify this.  
Brackets have been added to the formula.

9) The number of biological/ technical replicates should be mentioned.  
The technical replicates have been added in Figure 3.

10) I wonder whether the units in fig4A are correct. Do you really find only 0.01% crystalline cellulose in the cell wall (or biomass.... It is not clear what the "mg" is referring to) or should ng be µg?

The units have been changed to g/kg to be consistent with figure 3A. Based on our GC result, around 0.01% crystalline cellulose was measured in biomass.

11) As for all pictures and graphs, Fig4 (at least panel A) was published before, but that's not the point here. I have a problem with panel B. Not only is the variation extremely high, making it impossible to make the strong conclusion/statement made by the authors, that there is no effect on hemicellulose. Another issue of particular concern is the fact that these data are different from previously published values. In the original paper variation are much smaller.

The authors have replaced the Figure 4B to the previous one. Thank you for the correction.

12) It is not clear whether the different samples tested are different plants, different lines, different individuals, transformed with different construct... and what is the difference between "b" and "c" in the nomenclature.

The authors generated >30 1st generation (T0) transgenic maize. Among them, 3 transgenic lines showed significant ZmCCR1 transcript down-regulation and the lines have been annotated as 1a, 1b, and 1c. The progenies (T1) produced from T0 plants have been annotated as 1a-#, 1b-#, and 1c-#.

13) Rather than recycling figures, a new figure describing the cloning strategy could be added.   
More cloning details have been added to Figure 1.

14) The first step of the bombardment needs some more explanation for a technical paper. "subculture and grow highly proliferating immature-embryo-derived Hi-II embryonic maize calli..." is rather abstract if you are not familiar with tissue cultures. How do you do this? medium? Conditions? Handling?

The authors appreciate the comments and we decided to remove the subculture part to put more focus on the bombardment procedures. Thank you.