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## Methods to efficiently make homo- and hetero-grafts between watermelon and bottle gourd for the study of cold-responsive miRNAs --Manuscript Draft--

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Corresponding Author:	Pei Xu zhejiang Academy of Agricultural Sciences Institute of Vegetables hangzhou, CHINA
Corresponding Author's Institution:	zhejiang Academy of Agricultural Sciences Institute of Vegetables
Corresponding Author E-Mail:	peixu@mail.zaas.ac.cn
Order of Authors:	Lingping Wang Guojing Li Xiaohua Wu Dehui Qin Ye Tao Pei Xu
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Dear Editor:

Upon an invitation from JoVE, we would like to submit the enclosed manuscript entitled “Methods to study cold-responsive miRNAs in the watermelon-bottle gourd hetero-grafting system”. No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

In this work, we present a detailed protocol for making homo- and hetero-grafts between the cold-susceptible watermelon and the cold-tolerant bottle, in addition to methods of tissue sampling, data generation and analyzing. We believe the presented fits well the scope of JoVE and will be of broad interest to plant scientists.

Thank you and best regards.

Sincerely

Pei Xu

**TITLE:**

Generating Homo- and Heterografts Between Watermelon and Bottle Gourd for the Study of Cold-responsive microRNAs

**AUTHORS & AFFILIATIONS:**

Lingping Wang<sup>1</sup>, Guojing Li<sup>1,2</sup>, Xiaohua Wu<sup>1</sup>, Dehui Qin<sup>3</sup>, Ye Tao<sup>4</sup>, Pei Xu<sup>1,2</sup>

<sup>1</sup>Institute of Vegetables, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

<sup>2</sup>State Key Lab Breeding Base for Sustainable Control of Plant Pest and Disease, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

<sup>3</sup>Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

<sup>4</sup>Shanghai Biozeron Biotechnology Co., Ltd, Shanghai, China

**E-mail Addresses of the Co-authors:**

Lingping Wang (wanglingping@mail.zaas.ac.cn)

Guojing Li (ligj@mail.zaas.ac.cn)

Xiaohua Wu (Wuxh@mail.zaas.ac.cn)

Dehui Qin (qindh@mail.zaas.ac.cn)

Ye Tao (taoye@biozeron.com)

**Corresponding Author:**

Pei Xu (peixu@mail.zaas.ac.cn)

Tel: (86)-571-86402711

**KEYWORDS:**

bottle gourd; cold stress; differential expression; graft; miRNA; watermelon

**SUMMARY:**

Here we present a detailed protocol for efficiently making homo- and heterografts between watermelon and bottle gourd, in addition to methods of tissue sampling, data generation, and data analysis, for the investigation of cold-responsive microRNAs.

**LONG ABSTRACT:**

MicroRNAs (miRNAs) are endogenous small non-coding RNAs of about 20 - 24 nt, known to play important roles in plant development and adaptation. There is an accumulating evidence showing that the expressions of certain miRNAs are altered when grafting, an agricultural practice commonly used by farmers to improve crop tolerance to biotic and abiotic stresses. Bottle gourd is an inherently climate-resilient crop compared to many other major cucurbits, including watermelon, rendering it one of the most widely used rootstocks for the latter. The recent advancement of high-throughput sequencing technologies has provided great opportunities to investigate cold-responsive miRNAs and their contributions to heterograft advantages; yet, adequate experimental procedures are a prerequisite for this purpose. Here, we present a detailed protocol for efficiently generating homo- and heterografts between the cold-susceptible watermelon and the cold-tolerant bottle gourd, in addition to methods of

tissue sampling, data generation, and data analysis. The presented methods are also useful for other plant-grafting systems, to interrogate miRNA regulations under various environmental stresses, such as heat, drought, and salinity.

## INTRODUCTION:

Grafting has long been employed as an agricultural technique to improve crop production and tolerance to biotic and abiotic stresses<sup>1-3</sup>. In heterografting systems, elite rootstocks can enhance water and nutrients uptake of plants, strengthen resistance to soil pathogens, and limit the negative effects of metal toxicity<sup>4,5</sup>, which may confer the grafts an enhanced growth vigor and increased tolerance to environmental stresses. In many cases, heterografting can also impact fruit qualities in horticultural plants, leading to improved fruit flavor and increased content of health-related compounds<sup>6,7</sup>. It has been found that the long-distance transfer of phytohormones, RNAs, peptides, and proteins between the rootstock and the scion is a fundamental mechanism modulating the growth and development reprogramming of scion plants<sup>8,9,10</sup>. Grafting has been widely used in studies of long-distance signaling and transport in relation to environmental adaptation<sup>11</sup>. Grafting experiments are especially powerful for unambiguous detection of transmitted molecules in receiving tissue or vascular sap, and activation or suppression of molecular targets due to signal transmission<sup>12</sup>.

Non-coding RNAs, a big class of RNA that exert important regulatory functions in cells, have been reported to play a role in facilitating plant adaptation to abiotic stress<sup>13</sup>. miRNAs are endogenous small non-coding RNAs of about 20 - 24 nt. Studies have revealed the regulatory role of miRNAs in various aspects of plant activities, such as shoot growth, lateral root formation<sup>14,15,16</sup>, nutrient uptake, sulfate metabolism and homeostasis<sup>17</sup>, and responses to biotic and abiotic stress<sup>18</sup>. Recently, the expression of miRNAs and their target genes were related to salt stress tolerance in heterografted cucumber seedlings<sup>19</sup>. In the intervariety grafts of grape, the responses of miRNA expression to drought stress were found to be genotype-dependent<sup>20</sup>.

The rapid development and decreasing cost of high-throughput sequencing technology have provided a great opportunity for the study of miRNA regulations in agronomical plants. Watermelon (*Citrullus lanatus* [Thunb.] Mansf.), an important cucurbit crop grown throughout the world, is susceptible to low temperatures. Bottle gourd (*Lagenaria siceraria* [Molina] Standl.) is a more climate-resilient cucurbit commonly used by farmers to graft with watermelon. The primary goal of the current study is to establish a standard, efficient, and convenient method for making heterografts between watermelon (*Citrullus lanatus* [Thunb.] Mansf.) and bottle gourd (*Lagenaria siceraria* [Molina] Standl). This protocol also provides a detailed experimental scheme and analytical procedures for the study of the regulation of miRNA expressions following grafting, which is useful for revealing the mechanisms underlying heterografting advantages.

The plant materials used in this study include the watermelon cultivar and the bottle gourd landrace. Watermelon cultivar is a commercial cultivar with high yield but susceptible to low temperatures. Bottle gourd landrace is a popular rootstock for grafting with watermelon,

cucumber, and bottle gourd, due to its excellent tolerance of low temperatures<sup>21</sup>.

## **PROTOCOL:**

### **1. Seed Sterilization and Germination**

1.1. For surface sterilization, soak the bottle gourd seeds in a 500-mL beaker filled with water at 58 °C with occasional stirring, until the water temperature drops to 40 °C.

1.2. Meanwhile, put 3 kg of peat soil into a nylon bag and, to sterilize it, autoclave it at 120 °C/0.5 MPa for 20 min.

1.3. Keep soaking the bottle gourd seeds for 4 - 5 h more with no stirring.

1.3.1. Once the water reaches room temperature, rinse the seeds 2x - 3x with distilled water.

1.3.2. Drain the excess water and allow the seeds to sprout in a gauze bag at 28 °C in a growth chamber in the dark.

1.3.3. After germination, sow the seeds into plastic pots (6 cm in diameter) filled with sterilized peat soil.

1.4. When the bottle gourd seedlings have developed two flattened cotyledons, repeat steps 1.1 - 1.3 with watermelon seeds.

Note: This time management ensures that the sizes of the scion and rootstock match well for successful grafting.

### **2. Seedling Growth and Grafting**

2.1. Grow the seedlings in a growth chamber with a 16-h light /8-h dark cycle, keeping the temperature at 28 °C during the day (light) and at 22 °C during the night (dark). Irrigate the seedlings by adding water 1x a day in the late afternoon.

2.2. Use the cut-grafting method<sup>22</sup> to make heterografts when the seedlings of the bottle gourd (rootstock) are at the one true-leaf stage and the cotyledons of the watermelon (scion) have emerged (not yet flattened).

2.2.1. Cut the hypocotyls of the watermelon seedlings at 2 - 3 cm below the cotyledons, and the top of the bottle gourd seedlings at the site immediately above the true leaves.

2.2.2. Use a toothpick to make a hole in the top of the trimmed bottle gourd seedlings. Insert the trimmed watermelon seedlings into the holes of the bottle gourd seedlings to make heterografts.

2.3. Use a similar method as presented in step 2.2 to make homografts.

Note: Homo- and heterografting combinations should always be made simultaneously (**Figure 1**), which, in this case, results in the following: watermelon/bottle gourd (WB, heterograft), watermelon/watermelon (WW, homograft) and bottle gourd /bottle gourd (BB, homograft).

[Place **Figure 1** here]

### 3. Postgrafting Management, Cold Treatment, and Sampling

3.1. Enwrap the grafted seedlings with transparent polyethylene bags to keep a relatively high humidity and maintain them for 7 d under environmental conditions of 16-h light/8-h dark cycles, keeping the temperature at 28 °C during the day (light) and at 22 °C during the night (dark).

3.2. Uncover the transparent polyethylene bags on the 7th day. Let the plants grow for an additional 7 - 10 days under the same conditions.

3.3. Divide the healthy uniform seedlings into two groups, one for cold treatment (stressed) and one for control (non-stressed). For the control group, leave the seedlings in the same growth chamber (at 28 °C) for an additional 48 h, while for the cold-stressed group, transfer the seedlings to a growth chamber with a constant temperature at 6 °C, with light/dark conditions as described in step 2.1.

3.4. Sample the leaves of the scion and the rootstock from the grafts (**Figure 1**). Freeze the samples immediately in liquid nitrogen and store them at -70 °C until use.

### 4. Library Preparation and High-throughput Sequencing

4.1. Transfer the frozen samples to a 2-mL microcentrifuge tube in liquid nitrogen.

4.2. Add a stainless-steel bead (5 mm in diameter) to each tube containing the tissues.

4.3. Homogenize the tissues to a fine powder using a bead mill homogenizer for 30 s.

4.4. For each grafting combination, take equal amounts (0.1 g) of ground sample from ten seedlings and mix them in a 10-mL centrifuge tube. Add an appropriate amount of guanidium hydrochloride reagent (**Table of Materials**) based on the manufacturer's suggestions corresponding to the tissue weight.

4.4.1. Remove genomic DNA contaminations by adding RNA-free DNase I to 150 U/mL at 37 °C for 1 h.

4.5. Determine the total RNA quantity on a microcapillary electrophoresis system to ensure the RNA integrity number > 7.0.

NOTE: A RIN > 7.0 ensures a high integrity of the RNA samples.

4.6. Prepare small RNA libraries using a commercial kit (**Table of Materials**) according to the manufacturer's instructions. Use 1 µg of total RNA per sample to initiate.

4.6.1. Thaw library normalization reagents and adapters according to the manufacturer's guidelines. Ligate small RNAs with the 5' and 3' adapters and elute and purify them. Then, reverse transcribe the 5' and 3' ligated small RNAs following the manufacturer's guidelines.

4.6.2. Perform PCR amplification according to the manufacturer's protocol. Assess the quality and quantity of the cDNA libraries using a microcapillary electrophoresis system.

4.6.3. Load 1 µL of an RNA library on a microcapillary electrophoresis system to ensure the RIN > 7.0.

4.7. Sequence the small RNA libraries on a high-throughput sequencing instrument as described elsewhere<sup>23</sup>.

## 5. miRNA and Target Gene Prediction

5.1. For each grafting combination, use the open source UEA sRNA workbench 2.4-plant version<sup>24</sup> to remove poor-quality sequences and to trim adaptor sequences from the raw reads. Discard sequences that are smaller than 18 nt or larger than 32 nt.

5.2. Compare the high-quality "clean" sequences to the open source Rfam 11.0 database to recognize and remove reads of rRNA, tRNA, snoRNA, and other snRNAs.

5.3. Align the remaining reads to the reference genomes using a short-read sequence alignment tool<sup>25</sup>. No mismatch is allowed in this step.

Note: The watermelon "97103" genome assembly V1<sup>26</sup> was used for alignment with the reads from the scion, and the bottle gourd "HZ" genome assembly V1<sup>27</sup> was used for reads from the rootstock.

5.4. Compare the remaining reads against known mature miRNAs in the open source miRBase 22.0<sup>28</sup>. Reads that are homologous to known miRNAs are classified as conserved miRNAs.

5.5. Compare the sequences that fail to match the known miRNA precursors with the genome sequence. Use the MIREAP<sup>29</sup> algorithm to detect potential novel miRNAs under default settings.

## 6. Differential Expression and Gene Ontology Analysis

6.1. Compare the expression levels of miRNAs based on their read counts. miRNAs with a *P*-value (Fisher's exact test) < 0.05 and an absolute log<sub>2</sub> value > 2 are considered to be differentially expressed.

6.2. Use an antisense oligonucleotide target site selection tool (TargetFinder)<sup>30</sup> to predict potential complementary mRNAs (miRNA target genes) to the differentially expressed miRNAs under default parameters.

6.3. Use the gene ontology (GO) enrichment analytical tool<sup>31</sup> to reveal the miRNA target genes ontology (GO) patterns under a *P*-value threshold of 0.05 for statistical significance.

### REPRESENTATIVE RESULTS:

[Place **Figure 2** here]

Using the described method, we obtained a high success (survival) rate of 98% for grafting. Phenotypes of various grafts at room temperature and cold-stressed conditions are shown in **Figure 2**. After 48 h of cold treatment, the homografted watermelon plants showed obvious growth retardation with wilted young leaves, while the homografted bottle gourd plants and the watermelon/bottle gourd heterografts exhibited much more vigorous growth. No symptoms of damage were observed in the leaves of the heterografts, which even outperformed the homografted bottle gourd plants, where the lowest true leaves were damaged. These results clearly demonstrate the advantage of heterografts in conferring cold tolerance.

Small RNA sequencing of the eight libraries yielded a total of 258 million raw reads. After the quality control (QC), a total of 146 million reads corresponding to approximately 30 million unique sequences were retained (**Table 1**). Based on this set of clean sRNA sequences, 323 miRNAs, including 10 known and 313 novel miRNAs, were predicted from the bottle gourd, and 20 known and 802 novel miRNAs were predicted from the watermelon. sRNAs of 24 nt made up the biggest class of sRNAs in all grafting combinations, regardless of room temperature or cold-stressed conditions (**Figure 3**).

[Place **Table 1** here]

[Place **Figure 3** here]

Upon a 48-h of cold treatment, 30 and 268 miRNAs were up- and downregulated, respectively, in the leaves of the scion in the heterografts. This was in sharp contrast to the results in the leaves of rootstock, where 31 and only 12 miRNAs were up- and downregulated, respectively (**Figure 4**). In the watermelon/watermelon homografts, 64 and 83 miRNAs were up- and downregulated, respectively. In the bottle gourd/bottle gourd homografts, these numbers



were 30 and 28. Apparently, heterografting caused a profound reprogramming of the miRNA expressions. GO-enrichment analyses of the putative target genes of the differentially expressed miRNAs identified 78 enriched GO terms in the scion of the heterografts, with 40 classified into biological processes, 2 into cellular components, and 36 into molecular functions (**Figure 5**). We found that several known GO terms/pathways related to abiotic/biotic stress resistance and signal transduction, for instance, the chitin catabolic process (GO: 0006030, GO: 0006032), ethylene-activated signaling pathway (GO: 0009873), polyamine biosynthetic process (GO:0006596), and signal transduction by protein phosphorylation (GO: 0009755), were involved. Combined, our results suggest that the downregulation of miRNAs, by tuning the abundance of the transcripts of their target genes, may represent an important mechanism underlying enhanced cold tolerance. In the watermelon/bottle gourd grafts, the heterograft *per se* has a significant impact on the miRNA patterns that form the graft advantages.

[Place **Figure 4** here]

[Place **Figure 5** here]

#### **FIGURE & TABLE LEGENDS:**

**Table 1: Statistics of small RNAs in various grafts at room temperature or under cold treatment.**

**Figure 1: Illustration of the graft combinations and the grafted plant structures.** WB = watermelon/bottle gourd heterografting; WW = watermelon/watermelon homografting; BB = bottle gourd/bottle gourd homo-grafting; WB-S = scion leaves of the watermelon/bottle gourd heterografts that were sampled; WB-R = rootstock leaves of the watermelon/bottle gourd heterografts that were sampled.

**Figure 2: Phenotypes of various grafts at room temperature and cold-stressed conditions.** (a) This panel shows homo- and heterografted seedlings at room temperature as the control. (b) This panel shows homo- and heterografted seedlings after 48 h of cold treatment.

**Figure 3: Size distribution of the sRNA reads in various grafts.** (a) This panel shows the size distribution of the sRNA reads in heterografts under control or cold conditions. (b) This panel shows the size distribution of the sRNA reads in homografts under control or cold conditions.

**Figure 4: Comparison of the patterns of up- and downregulated miRNAs in response to cold stress in various grafts.** WB-S = scion leaves of the watermelon/bottle gourd heterografts that were sampled; WB-R = rootstock leaves of the watermelon/bottle gourd heterografts that were sampled; WW = the watermelon/watermelon homografts; BB = the bottle gourd/bottle gourd homografts.

**Figure 5: GO enrichment analyses of the putative target genes of differentially expressed miRNAs in the scion leaves of heterografts upon cold stress.** WB-CL-S = scion leaves of the watermelon/bottle gourd heterografts under cold treatment; WB-CK-S = scion leaves of the watermelon/bottle gourd heterografts at room temperature.

## **DISCUSSION:**

In this protocol, we described in detail a highly efficient and reproducible method to make homo- and heterografts between watermelon and bottle gourd. This method, requiring no specific equipment, is very easy to operate and typically has a very high survival rate of grafting. The method can also be used to make grafts for other cucurbits, such as between watermelon, cucumber, and pumpkin.

It is worth noting that the relative size (age) of the rootstock and scion is critical to making a successful graft (step 2.2 of the **Protocol**). We observed that, if the rootstock used was too large compared to the scion, the graft union was more difficult to form because the stem of the scion was somewhat hollowed. Based on our previous proteomic data<sup>31</sup>, the inclusion of self-grafted scion and self-grafted rootstock as controls is strongly recommended (step 2.3 of the **Protocol**), because then, the impact of grafting injuries can be largely eliminated.

This protocol also provides a detailed experimental scheme and specific experimental procedures for investigating the abundances of miRNAs in the heterografting system. This method will also be useful for studies in other plant-grafting systems to reveal the mechanisms of local and long-distance miRNA regulation. In the **Representative Results**, we report the expression changes of only local miRNAs in the scion or rootstock in response to a low temperature. Accumulating reports have highlighted the involvement of long-distance small RNA transmission in grafting-related phenotypic changes. The protocol presented here, which combines the methods for grafting and high-throughput data analysis, can also be used for miRNA transmission analysis between the scion and the rootstock. The principle for differentiating transmitted miRNAs from local miRNAs is based on their sequence similarity to the reference genomes (*i.e.*, a miRNA in the scion that is more like the rootstock genome is considered to be transferred from the rootstock, and *vice versa*).

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## **DISCLOSURES:**

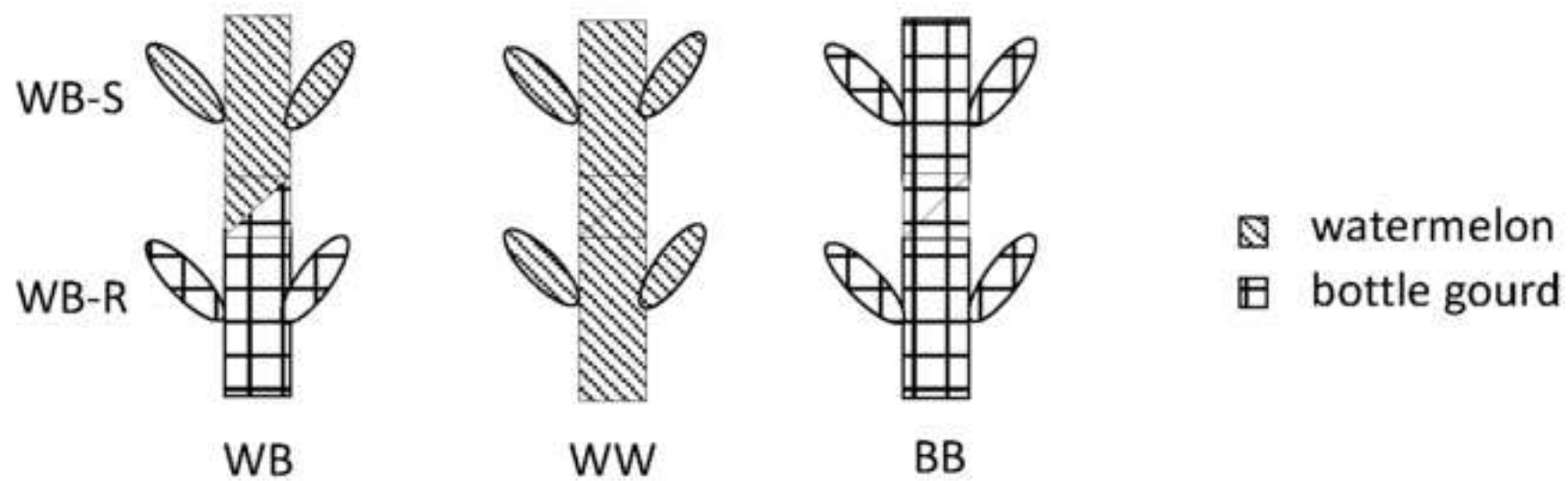
The authors have nothing to disclose. The small RNA sequencing data is deposited in the GenBank under the accession number SRP136842.

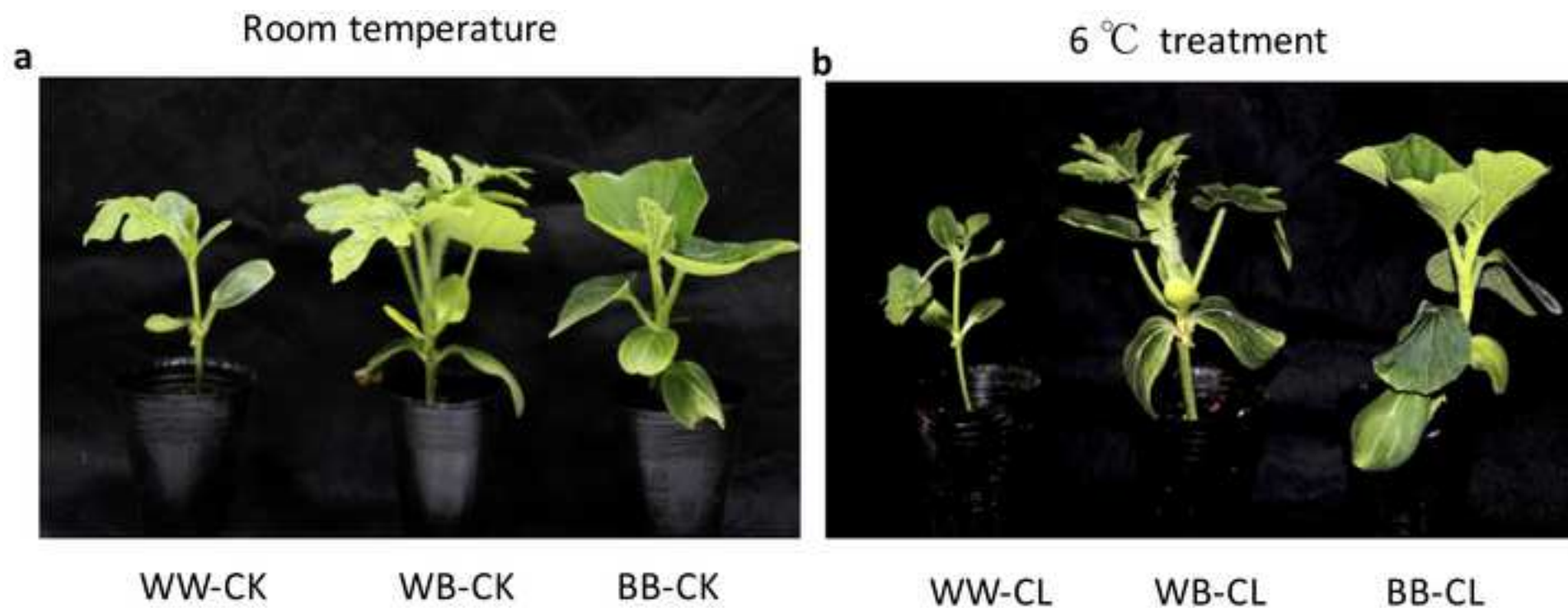
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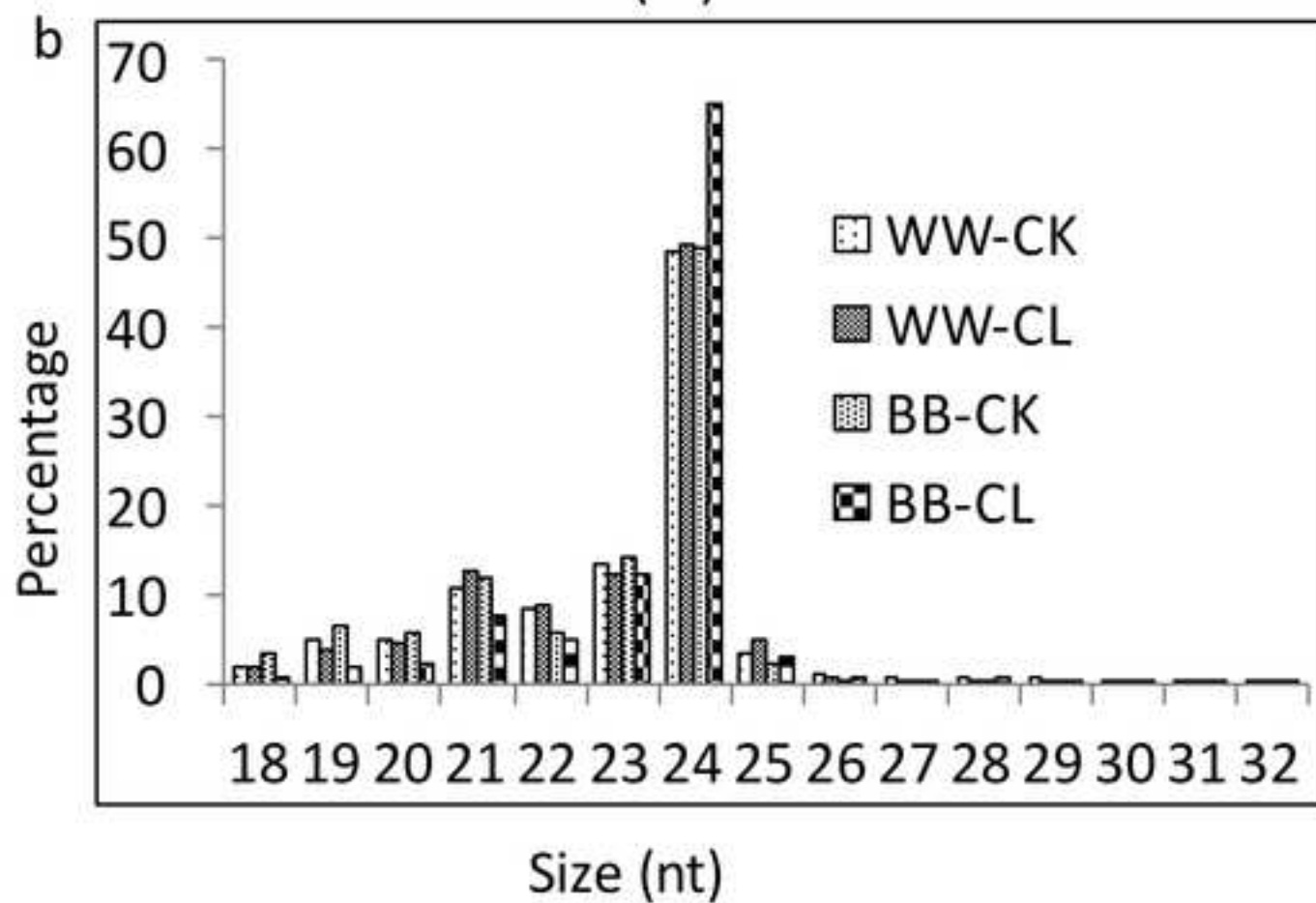
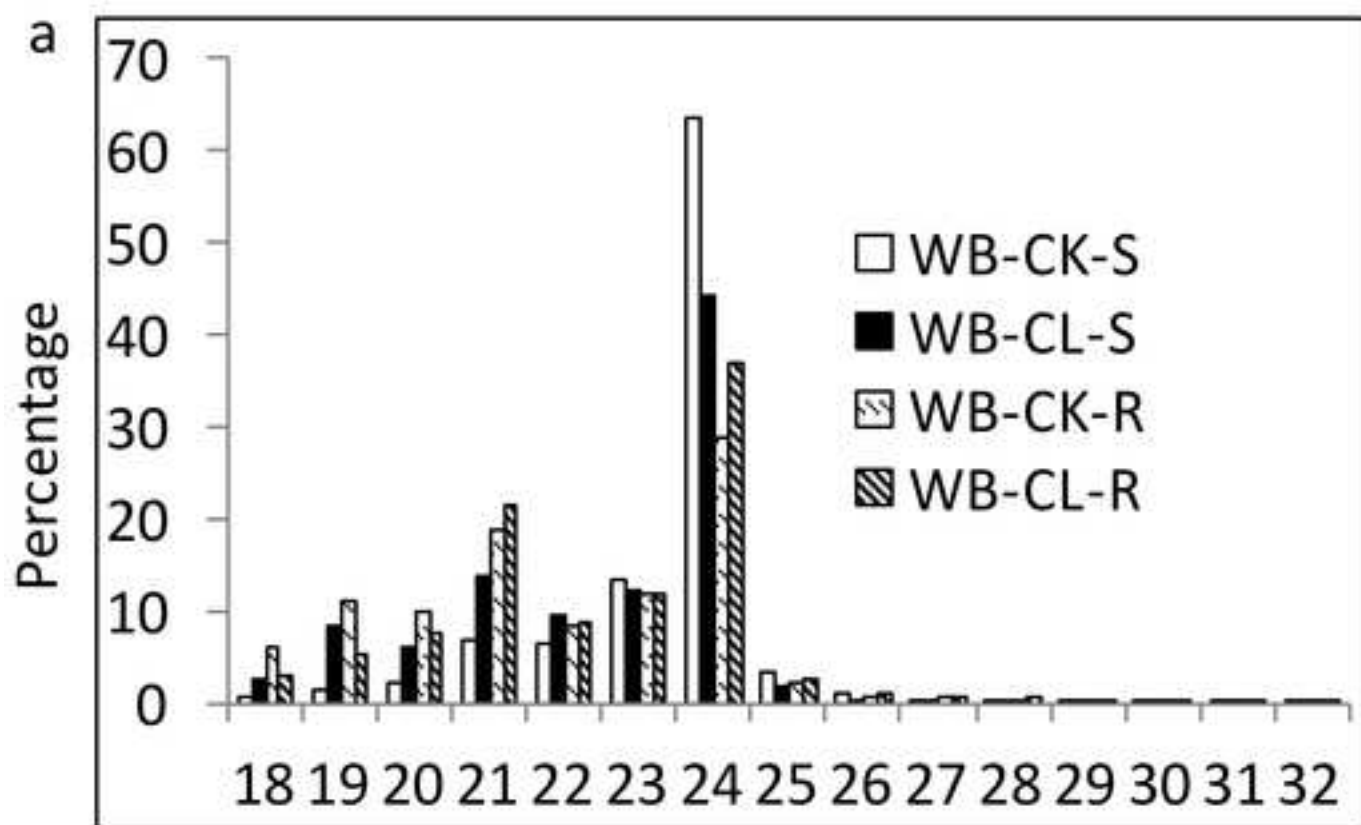
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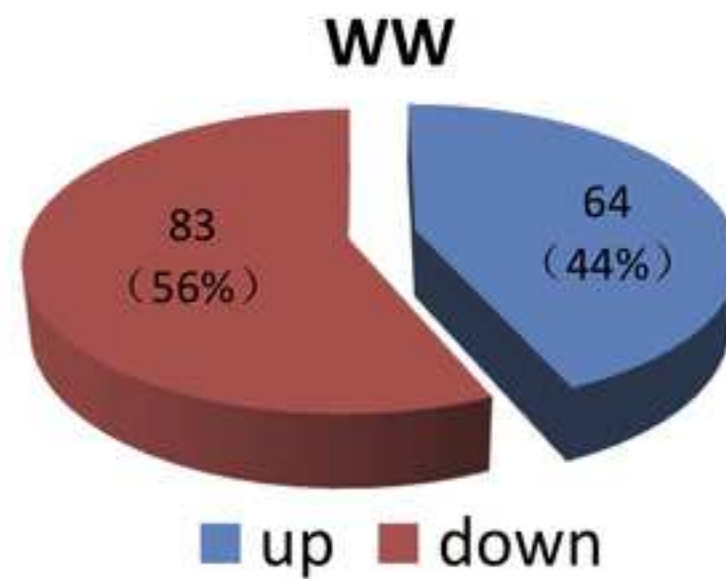
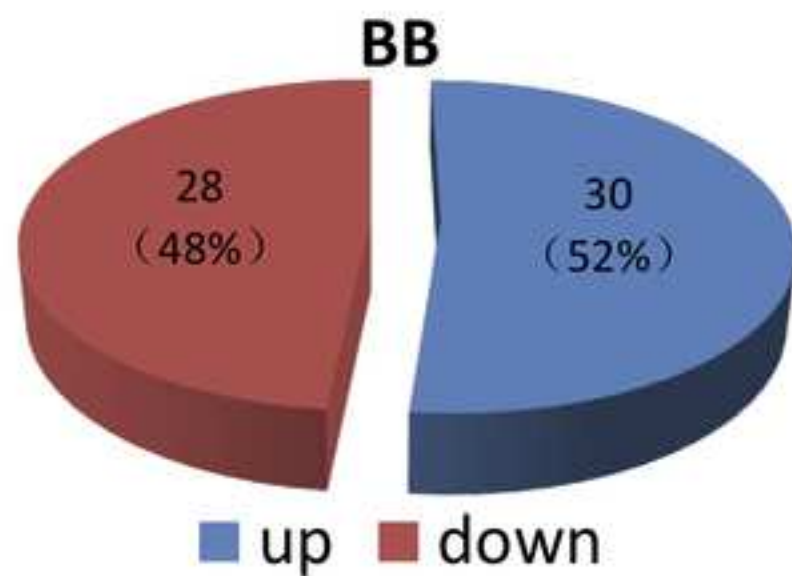
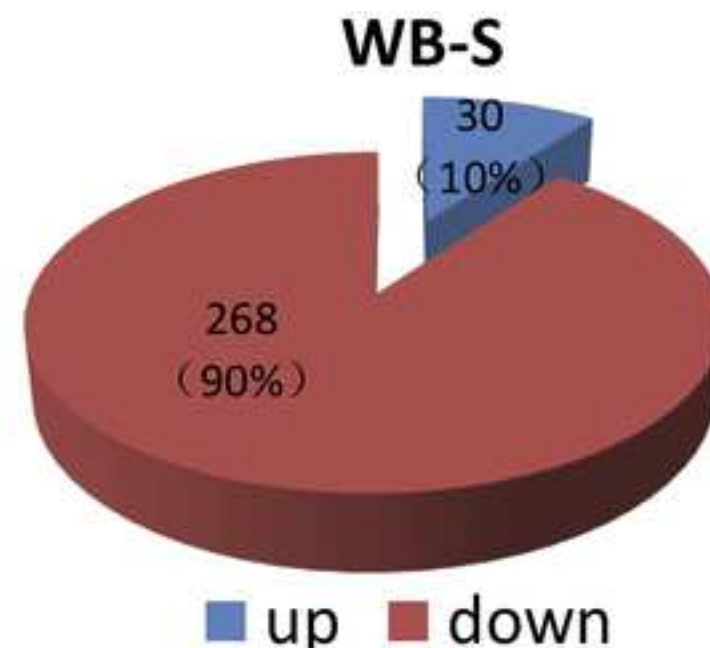
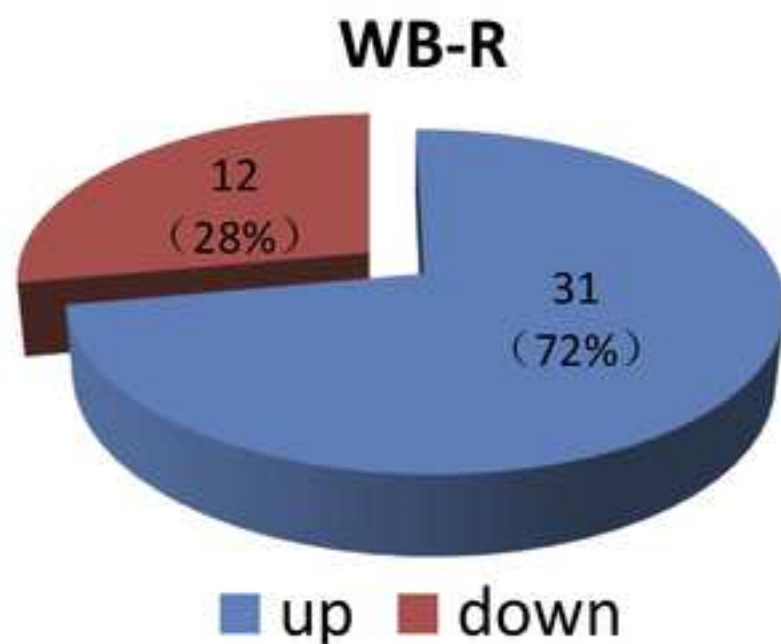
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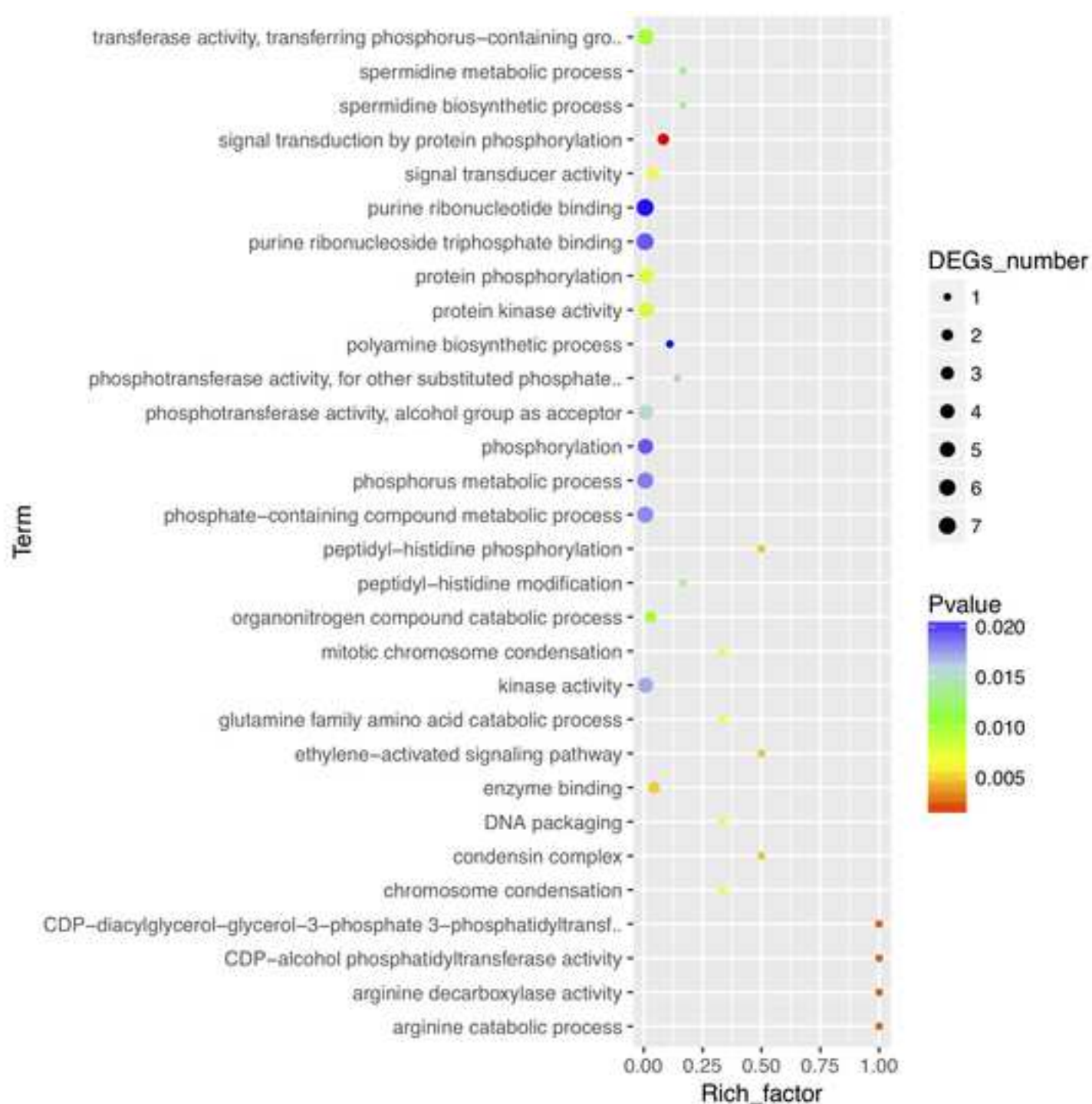


Table 1 Statistics of small RNAs in various grafts under room temperature or cold treatment

Treatment	Code	No. reads	sRNA	
			Total	Unique
CK	WW-CK	Raw	30612962	
		Clean	19727501	3858868
		Mapped to genomic	19059359	3777952
	BB-CK	Raw	30845546	
		Clean	16832061	3787866
		Mapped to genomic	16375142	3694388
	WB-CK-S	Raw	39492123	
		Clean	26783053	6319473
		Mapped to genomic	25919944	6132389
	WB-CK-R	Raw	23763619	
		Clean	10187791	1784447
		Mapped to genomic	8946929	1537867
Cold	WW-CL	Raw	27557577	
		Clean	17879038	3336242
		Mapped to genomic	17153763	3259960
	BB-CL	Raw	29780991	
		Clean	13342206	3235570
		Mapped to genomic	12949972	3164329
	WB-CL-S	Raw	45708415	
		Clean	23071845	4310276
		Mapped to genomic	22363113	4224166
	WB-CL-R	Raw	30585408	
		Clean	19029266	3541729
		Mapped to genomic	17364239	3196106

Name of Material/ Equipment				Company	Catalog Number	Comments/Description
TRIzol Reagent				Invitrogen	15596026	
RNA-free DNase I				Takara	D2270A	
Truseq Small RNA sample prep Kit				Illumina	RS-200-0012	
2100 Bionalyser				Agilent	5067	
DNA Polymerase				Thermo Scientific	Fisher F530S	
UEA	sRNA	workbench	2.4-plant	NA	NA	http://srna-
version (software)						workbench.cmp.uea.ac.uk/
Rfam 11.0 database (website)				NA	NA	http://rfam.janelia.org
miRBase 22.0 (website)				NA	NA	http://www.mirbase.org/
MIREAP(software)				NA	NA	https://sourceforge.net/projects/mireap/
TargetFinder (software)				NA	NA	http://targetfinder.org/





1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
[www.jove.com](http://www.jove.com)

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Methods to study cold-responsive miRNAs in the watermelon-bottle gourd hetero-grafting system

Author(s):

Lingping Wang, Guojing Li, Dehui Qin, Xiaohua Wu, Ye Tao, Pei Xu

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## Point-by-Point Responses to Editor' Comments

We wish to thank editor for his/her support and valuable comments on this work.

### Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

**Response:** Done as requested.

2. Please remove the embedded Table 1 from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

**Response:** Done as requested.

3. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

**Response:** Done as requested.

4. Figure 2: Please fix the temperature symbol (the degree symbol should be a superscript) and correct the typo “treatment” in panel b.

**Response:** Done as requested

5. Figure 2/3/4: Please line up the panels better. Many of the panels are off-set. Please ensure that the panels are of the same dimensions if possible. Please include a space between “Size” and “nt” in Figure 3.

**Response:** Done as requested

6. Figure 4: Please also define WW and BB treatments in figure legend.



**Response:** We have defined WW and BB in figure 4 legend as requested.

7. Figure 5: Please ensure that figure legend correctly describes the figure. The defined terms in figure legend are not seen in Figure 5. Also please submit the figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.

**Response:** Thanks for pointing this out. We have corrected Figure 5 and re-formatted the figures as requested.

8. Keywords: Please provide at least 6 keywords or phrases.

**Response:** One more key word “cold stress” was added.

9. Please revise the Introduction to include all of the following:

- a) A clear statement of the overall goal of this method
- b) The rationale behind the development and/or use of this technique
- c) The advantages over alternative techniques with applicable references to previous studies
- d) A description of the context of the technique in the wider body of literature
- e) Information to help readers to determine whether the method is appropriate for their application

**Response:** we have revised the introduction in taking account of all the advices above.

10. Please include a space between all numbers and their corresponding units: 58 °C, etc.ok

**Response:** Done as requested

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Retsch ball mill MM400, Trizol reagent, Takara, Agilent, TruSeq, SuperScript II Reverse Transcriptase, Illumina Hiseq2500,

UEA sRNA workbench 2.4-plant version (<http://srna-tools.cmp.uea.ac.uk>), etc.

**Response:** Done as requested

12. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

13. 1.3: How to sterilize peat soil?

**Response:** add a step 1.3 “Put peat soil into bag and 120 °C for 20 minutes to sterilize” .

14. 3.1: How to maintain relative humidity in the bag?

**Response:** we have revised this sentence. please see step 3.1.

15. 4.2: Please specify the amounts of grounded sample.

**Response:** the amount 0.1 g was specified. see step 4.2.

16. Please use the Greek symbol  $\mu$  for the micron abbreviation throughout (see 4.4).

**Response:** Done as requested.

17. 4.4: Please break up into substeps. Add more details if possible.

**Response:** Contents of the original 4.4 was divided into three substeps from 4.4 to 4.6.

18. 4.5: Please provide a reference for how to perform this step.

**Response:** A new reference was added.

19. For steps 5 and 6, please do not include them for filming. Please provide citations for the analysis in these steps.

**Response:** Yes, we understand that steps 5 and 6 will not be included for filming. Eight references related to the analysis in these steps were added.

20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

**Response:** we have revised the Discussion by taking account of all the advices above.

21. Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

**Response:** we have carefully corrected the formats of the references according to the journal guidelines.

## Point-by-Point Responses to the Reviewers' Comments

We wish to thank reviewer for his/her support and valuable comments on this work.

### Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript authored by Wang et al describes a methodology for creating homo and heterografts between watermelon cultivar "8424" and the bottle gourd landrace "Y1". Further, it explains the changes in miRNA expression pattern that was ascertained using Next Generation Sequencing (NGS) platforms. Overall the manuscript was well presented however, I feel there some concerns that need to be addressed to merit a publication.

Major Concerns:

There is nothing new to create a homo or hetero grafts of watermelon and bottle gourd. Similarly use of NGS based deep sequencing efforts to obtain small RNA expression data is being carried out in most of the crops. Hence, I don't see any valid reason for this manuscript to warrant a publication in Journal of visualized experiments

**Response: JoVE is a methods journal, and the current MS was made upon invitation of the journal editor.**

Minor Concerns:

If Editor decides the work presented in this manuscript falls well within the scope of journal then following revisions are suggested

a) The word "overperforming" in abstract needs to be changed. Though writing has been good. There are instances of incorrect words.

**Response: This sentence has been reparsed. Incorrect wording throughout has been carefully corrected.**

b) abstract states that " The presented methods are easily transferable to other plant

grafting systems to interrogate miRNA regulations under various environmental stresses such as heat, drought and salinity" but authors provide neither experimental proof of their own or published information to support this claim. Better to reword the sentence

Response: we have rephrased the sentence to "The presented methods are also useful for other plant grafting systems to interrogate miRNA regulations under various environmental stresses such as heat, drought and salinity".

c) No validation of miRNA expression, following deep sequencing, was presented in the manuscript. Especially quantitative real-time PCR (RT-qPCR) based validation of few select miRNAs could have been better and more convincing. In the absence of this data the results and interpretation is not convincing.

Response: We thank the reviewer for this suggestion. Yes, including RT-qPCR validation of miRNAs expression could have been better. However, as a method article dedicated to presenting the technical details of our hetero-grafting methods, the current MS does not include this part.

d) Figure 1 is unnecessary and at the best could be described as a text-book material does not suit scientific publication hence better to remove it

Response: Given that JoVE is a methods journal, Figure 1 is considered useful in this article.

Reviewer #2:

Thank you for your support and valuable comments on this work !

The title is satisfactory.

Abstract:

The abstract is satisfactory and in some how, Does not characterize the essence of the work.

Suggestions and Comments?

Expand the key results and include a solid take home message.

The key words are adequate.

**Response:** Thanks for the reviewer's advices. The abstract has been improved to characterize the essence of the work.

Introduction:

Adequate

Inadequate and explicit hypothesis to be tested is missing

Suggestions and Comments? Not clear what is known and what not, and what is new and novel in this study. Testing hypothesis / clear objectives are lacking.

The problem is well stated.

**Response:** Thanks for the valuable comments. The Introduction has been improved according to the comments. Specially, the issues that " Not clear what is known and what not, and what is new and novel in this study " was addressed.

Methods:

The description and documentation is improbable. Like all the protocols is included, it's too much.

The experiment is properly planned. The data is reliable.

The conclusion should be adjusted.

The discussion could be improved.

Response: Thanks for the valuable comments. The conclusion has be adjusted and the discussion improved.