

1 **MiRNA expression profiling and zeatin dynamic changes in**
2 **a new model system of *in vivo* indirect regeneration of**
3 **tomato**

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12 **Abstract**

13 Callus formation and adventitious shoot differentiation could be observed on the cut
14 surface of completely decapitated tomato plants. We propose that this process can be
15 used as a model system to investigate the mechanisms that regulate indirect
16 regeneration of higher plants without the addition of exogenous hormones. This study
17 analyzed the patterns of trans-zeatin and miRNA expression during *in vivo*
18 regeneration of tomato. Analysis of trans-zeatin revealed that the hormone cytokinin
19 played an important role in *in vivo* regeneration of tomato. Among 183 miRNAs and
20 1168 predicted target genes sequences identified, 93 miRNAs and 505 potential

21 targets were selected based on differential expression levels for further
22 characterization. Expression patterns of six miRNAs, including sly-miR166,
23 sly-miR167, sly-miR396, sly-miR397, novel 156, and novel 128, were further
24 validated by qRT-PCR. We speculate that sly-miR156, sly-miR160, sly-miR166, and
25 sly-miR397 play major roles in callus formation of tomato during *in vivo* regeneration
26 by regulating cytokinin, IAA, and laccase levels. Overall, our microRNA sequence
27 and target analyses of callus formation during *in vivo* regeneration of tomato provide
28 novel insights into the regulation of regeneration in higher plants.

29 **Keywords:** Cytokinin, Callus formation, Organogenesis, Tissue culture, Tomato

30

Introduction

31 Tissue culture established over 150 years ago continues to play an important role in
32 plant propagation, and continues to be utilized in both basic and applied plant
33 research, including gene transformation and molecular breeding [1,2]. In-depth
34 studies into mechanisms of regulation of regeneration of higher plants using *in vitro*
35 culture techniques, identified several proteins and transcription factors such as
36 WUSCHEL (WUS), SHOOT MERISTEMLESS (STM), BABY BOOM (BBM), and
37 MONOPTEROS (MP) [3–6]. However, both direct regeneration and indirect
38 regeneration via an intermediate callus phase are introduced by various plant growth
39 regulators supplemented media in traditional tissue culture. Yin[7] reported that 157
40 unique proteins were significantly differentially expressed during callus

41 differentiation in rice when treated with different relative concentrations of the
42 hormones cytokinin and auxin. Additionally, even though somatic embryogenesis
43 (SEG) has been proposed to be a model system of plant embryogenesis, the
44 expression of gene families such as those of MIR397 and MIR408 was detected in
45 somatic embryos (SE), but greatly decreased in zygotic embryos (ZE) in conifer
46 species[8].

47 An interesting phenomenon has been observed that *in vivo* adventitious shoots can be
48 regenerated from cut surfaces of stems or hypocotyls after removal of both apical and
49 axillary meristems in some species such as *Cucurbita pepo*[9], tomato[10], and
50 poinsettia [11]. In tomato, the surface of cut stems regenerates plenty of shoots via
51 callus formation. This *in vivo* generation does not depend on the presence of
52 exogenous hormones. We propose that this phenomenon is particularly useful as a
53 model system to study the innate molecular mechanisms of plant regeneration

54 MicroRNAs (miRNAs) are a class of small noncoding-RNAs (20–24 nt) that regulate
55 gene expression at post-transcriptional levels by directly binding to their
56 targets[12,13]. In the past 20 years, miRNAs have been shown to play key roles at
57 each major stage of plant development [14–17]. Furthermore, recent studies have
58 shown that miRNAs are involved in callus initiation, formation and differentiation.
59 For example, the expression levels of miR408, miR164, miR397, miR156, miR398,
60 miR168, and miR528 were up-regulated during maize SE induction[18]. Another
61 study demonstrated that over-expression of miR167 inhibited somatic embryo

62 formation by inhibiting the auxin signaling pathway in *Arabidopsis*[19]. In citrus, the
63 ability of the callus to form SEs was significantly enhanced by either over-expression
64 of csi-miR156a or by individual knock-down of its two target genes, *CsSPL3* and
65 *CsSPL14*[20].

66 Recently, the large scale application of next-generation sequencing has proved to be a
67 useful tool to identify the patterns of miRNA expression during plant regeneration.
68 Genome-wide miRNAs and their targets have been analyzed during explant
69 regeneration in vitro in wheat[21] , rice[22,23], cotton[24], peanut[25], sweet orange
70 [26], coconut[27], larch[28], maritime pine[8], Norway Spruce[29] , longan[30],
71 yellow-poplar[31], radish[32], *Lilium* [33], and Tuxpeno maize[34]. However, all
72 these studies were performed on *in vitro* specimens, which relies on the presence of
73 exogenous hormones to regenerate plantlets. The aim of the present study was to
74 identify the pattern of miRNA expression during callus formation in *in vivo*
75 regeneration in tomato through sequencing. We assessed 92 known miRNAs and
76 identified 91 novel miRNAs, of which several were found to be developmentally
77 regulated. We also analyzed dynamic changes in cytokinin levels during *in vivo*
78 regeneration of tomato.

79 **Materials and Methods**

80 **Plant materials**

81 The tomato cultivar micro-TOM was used in this study. Seeds were placed on

82 moistened filter papers for approximately 3 to 4 d until the seeds sprouted. The
83 germinated seeds were seeded into a tray of 72 cells filled with a mixture of nutrient
84 soil, matrix, vermiculite and perlite (2:2:1.5:0.5(v/v/v/v)), and grown in a culture
85 room with temperature ranging from 23 to 28 °C and 16/8 h light/dark photoperiod.
86 When the seedlings had grown 6 to 8 true leaves, the primary shoot was decapitated
87 horizontally. All axillary buds that appeared after decapitation were resected at the
88 base.

89 **HPLC analysis of trans-zeatin**

90 Cutting surfaces of stems (3 mm long) were sampled at 0, 9, 12, 15, 18, 21, 24 and 30
91 d after decapitation. The samples were immediately frozen in liquid nitrogen and
92 stored at -80 °C. Trans-zeatin was extracted with 80% methanol from samples and
93 analyzed using HPLC (Agilent 1100) connected to an UV detector ($\lambda = 274$ nm). The
94 passing fraction was further purified by Sep-pak C18 column (Waters). Gradient
95 elution was with a mixture of water-methanol (75:25 (vol:vol)) with an elution rate of
96 1.0 mL/ min at a column temperature of 35 °C. The absorbing material was Agilent
97 C18 with a particle size of 5 μ m loaded into a stainless steel column (250 \times 4.6 mm).

98 **Lovastatin addition during the tomato regeneration in vivo**

99 Lovastatin (Sigma-Aldrich, USA) dissolved in DMSO (stock solution: 0.124 M) and
100 the final concentrations of 123.6 μ M was applied. 20 μ L lovastatin was dripped on
101 cutting surfaces of 10 tomato stems after decapitation with 1 μ L Tween-20. Another

102 10 tomato plants were treated with water as control.
103 A lovastatin (Sigma-Aldrich, USA) stock solution at a concentration of 0.124 M was
104 prepared by dissolving in DMSO and applied at a final concentration of 123.6 μ M. A
105 solution of 20 μ L lovastatin and 1 μ L Tween-20 was applied on the cut surfaces of 10
106 tomato stems after decapitation. An additional equal number of tomato plants were
107 treated with water as control.

108 **sRNA library construction and RNA sequencing**

109 Total RNA was extracted from decapitated stem at 0 and 15 d in three biological
110 replicates. Each biological replicate was from a pool of 8–10 tomato plants. RNA
111 samples of the three biological replicates were mixed in equal amount and used for
112 the construction of libraries. The small RNA library was constructed using 3 μ g total
113 RNA from each treatment respectively as input materials. The sequencing library was
114 generated by NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB,
115 USA), with added index codes to attribute sequences of each sample as recommended
116 by the manufacturer. Briefly, the NEB 3' SR Adaptor was connected to 3' end of
117 miRNA, siRNA and piRNA directly. After the 3' ligation reaction, the single-stranded
118 DNA adaptor was transformed into a double-stranded DNA molecule by
119 hybridization of the SR RT Primer with excess of 3' SR Adaptor (kept free after the 3'
120 ligation reaction). This step significantly reduced the formation of adaptor-dimers. In
121 addition; dsDNAs were not the T4 RNA Ligase 1-mediated-substrates, and therefore

122 were not ligated to the 5' SR Adaptor in the following ligation step. The 5' ends
123 adapter was connected to the 5' ends of miRNAs, siRNA, and piRNA. Reverse
124 transcription reaction was performed using M-MuLV Reverse Transcriptase (RNase
125 H⁻) after ligation with adapters, and Long Amp Taq 2X Master Mix, SR Primer for
126 Illumina and index (X) primer was used for PCR amplification. An 8%
127 polyacrylamide gel was used for purifying PCR products, small RNA fragments
128 approximately 140–160 bp were recovered and dissolved in elution buffer. Finally,
129 the quality of library was evaluated on the 2100 system of Agilent Bioanalyzer using
130 DNA High Sensitivity Chips. TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San
131 Diego, CA, USA) was used to evaluate index-coded samples on a cBot Cluster
132 Generation System. After clustering, the library preparations were sequenced on an
133 Illumina Hiseq 2500 platform, and 50 bp single-end reads were generated.

134 **MiRNA identification and target prediction**

135 All sequenced data were firstly filtered with the removal of N% >10% reads, length
136 <18 nt or >30 nt, with 5' adapter contamination, 3' adapter null or insert null and low
137 quality reads to obtain clean reads. The remaining clean reads were mapped to the
138 reference sequence by Bowtie to obtain unique reads and analyze the length
139 distribution and expression of unique sRNAs. Unique reads were mapped to miRNA,
140 rRNA, tRNA, snRNA, snoRNA, repeat masker, NAT, TAS, exon, intron, and others.
141 Mapped sRNA tags were used to search for known miRNAs. With miRBase20.0 as

142 reference, the potential miRNAs were identified using modified software mirdeep2
143 and srna-tools-cli, and then the secondary structures were drawn. We used miREvo
144 and mirdeep2 to predict novel miRNAs through the precursor structure of each
145 miRNA unannotated in the previous steps, including the analysis of the secondary
146 structure, the dicer cleavage site, and the minimum free energy. We used miFam.dat
147 (<http://www.mirbase.org/ftp.shtml>) to compare our candidate miRNA families with
148 known miRNA families from other species.
149 We used psRobot_tar in psRobot to identify the potential gene targets of known and
150 novel miRNAs.

151 **Differential expression analysis of miRNAs**

152 We used the TPM (transcript per million) value to estimate the differential expression
153 levels of miRNAs between stem and callus[35]. The TPM ratio of miRNAs between
154 stem and callus libraries was computed as \log_2 (callus/stem). miRNAs with p value
155 <0.05 and \log_2 (callus/stem) <-1 or >1 were regarded to have as significantly
156 differential expression levels through the Bayesian method.

157 **MiRNAs quantification by qRT-PCR**

158 Primers were designed by stem-loop method to perform RT-PCR assays according to
159 Chen's design[36]. A 20 μ L final volume Reverse transcription (RT) reaction was
160 carried out to validate the expression levels of selected miRNAs extracted from stem
161 and callus respectively. The final 20 μ L reaction system included 1 μ L template, 1 μ L

162 stem-loop primer, 1 μ L dNTP mixture, 4 μ L buffer, 1 μ L reverse transcriptase and 0.5
163 μ L RNase inhibitor. In the reaction tube, stem-loop primer, dNTP mix and template
164 were added first, and then the template was denatured at 65 °C for 5 min to improve
165 the efficiency of reverse transcription. The tube was placed on ice to cool for 2 min,
166 followed by the addition of the buffer, reverse transcriptase and RNase inhibitor. The
167 tube was then incubated at 45 °C for 60 min, followed by 95 °C for 5 min. The
168 reverse transcription reaction was completed by cooling on ice for 2 min. After
169 reverse transcription, 1 μ L of the RT reaction mixture was used for PCR. The PCR
170 system was 25 μ L, containing 12.5 μ L PCR mix, 1 μ L template, 1 μ L downstream
171 primer and 1 μ L upstream primer, supplemented to 25 μ L with nuclease-free water.
172 The PCR conditions were as follows: 94 °C for 2 min, 94 °C for 30 s, 60 °C for 1 min
173 for 35 cycles, followed by a final extension of 72 °C for 5 min. Following the PCR
174 assay, gel electrophoresis was used to detect the amplified products.
175 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were
176 performed using a C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA, USA).
177 The reaction system included 5 μ L SYBR® Premix Ex Taq™ (Takara, China), 1 μ L
178 template, 0.2 μ L upstream primer, 0.2 μ L downstream primer and 3.6 μ L
179 nuclease-free water. The reaction conditions were as follows: 94 °C for 2 min, 94 °C
180 for 30 s, 60 °C for 1 min, and final extension at 72 °C for 5 min for 35 cycles. The
181 sequences of all primers used in this study are compiled in S1 Table.

182 **Results**

183 **Phenotypic analysis of *in vivo* regeneration**

184 In tomato, a primary shoot shows apical dominance and inhibits outgrowth of axillary
185 buds. After excising the main shoot apex, the dormant axillary buds began to develop
186 immediately to replace the lost shoot apex. Since all new axillary buds were excised,
187 light-green callus gradually formed at the cut surface of primary shoots and axillary
188 buds followed by progression to the compact and nodular stage with a maximum
189 diameter of up to 1 cm. When the callus entered its differentiation stage, a large
190 number of purple dots appeared on its surface, and finally the shoots appeared to
191 regenerate through callus (Fig. 1). It took 30 days to obtain macroscopic shoots after
192 decapitation at 25°C. These features of *in vivo* regeneration were similar to the
193 responses seen in tissue culture (Tezuka et al., 2011).

194 **Fig. 1. External appearance of the different stages of *in vivo* regeneration in**
195 **tomato.**

196 (A) The decapitated primary shoot; (B),(C) The callus formed on the cutting surface
197 at 15 and 25d after decapitation; (D) The adventitious shoots differentiated from
198 callus.

199 **Analysis of trans-zeatin during *in vivo* regeneration**

200 HPLC analysis of trans-zeatin during *in vivo* regeneration of tomato micro-TOM is

201 presented in Fig. 2. Trans-zeatin was not detected in 0 d stem, but was detected in
202 gradually increasing amounts correlated with the progress of callus initiation,
203 formation and differentiation. These results show that cytokinin plays a key role
204 during *in vivo* regeneration of tomato. These data correlated well with previous
205 studies showing that 6-benzyladenine treatment increased the number of adventitious
206 shoot amounts during *in vivo* tomato regeneration [37]. Furthermore, our findings are
207 also supported by other studies which used zeatin as the only exogenous hormone
208 during *in vitro* regeneration of tomato [38–40].

209 **Fig. 2. HPLC analysis of trans-zeatin levels during *in vivo* regeneration of tomato**
210 **micro-TOM.**

211 The samples were the cutting surface of stem at 0, 9, 12, 15, 18, 21, 24 and 30 d after
212 decapitation.

213 Cytokinins are a heterogenous group of N6-substituted adenine derivatives[41].
214 Lovastatin is a potent inhibitor of the mevalonate pathway, and in principle blocks the
215 synthesis of isopentenyl-pyrophosphate and inhibit the biosynthesis of cytokinin[42].
216 Lovastatin (1 μ M) has been shown to completely inhibit the growth of cultured
217 tobacco cells [43]. However, in this study, the addition of high levels lovastatin (123
218 μ M) to the cut surface of decapitated stems did not inhibit tomato regeneration *in vivo*.
219 There was no obvious difference in the number of regenerated adventitious shoots
220 between lovastatin and control treated plants. Together, these observations suggested
221 that cytokinin was not biosynthesized de novo in the cells at the cut surface or in the

222 callus during *in vivo* regeneration. Cytokinin is found in the xylem sap as a
223 long-distance signal in intact plants [44–46]. To date, the trans-zeatin is the major
224 form of cytokinin in xylem sap [47]. The trans-zeatin is produced mainly in the root,
225 and can then be transported from the root to shoot[48]. Therefore, we speculate that
226 cytokinin was also transported over long distances from the root to callus cells during
227 *in vivo* regeneration of tomato.

228 **Deep-Sequencing of sRNAs in stem and callus**

229 To study the vital role of miRNAs during *in vivo* regeneration, the cut surfaces of
230 stems at 0 and 15 days after decapitation were used to construct two sRNA libraries.
231 Both of these libraries were sequenced with 13.6 and 11.0 million raw reads obtained
232 from stem and callus libraries, respectively (S2 Table). After removal of the low
233 quality reads as described in the Materials and Methods section, 9.5 and 7.5 million
234 clean sRNAs were obtained from the stem and callus libraries, the stem and callus.
235 Among these, 7.4 and 6.6 million were unique reads aligned to the reference
236 sequences (Table 1). Unique sRNAs ranged from 18 to 30 nt in length in the two
237 libraries (Fig. 3). The most common lengths of unique sequences in each library were
238 21–24 nt, with 24 nt long reads being the majority, followed by 23 nt.

239 **Fig. 3. Length distributions of unique sRNAs in stem and callus.**

240 **Table 1. Sequencing data filtering of two sRNA libraries produced from stem**
241 **and callus.**

Type	Number of reads (Percentage of reads)	
	Stem	Callus
Raw reads	13,563,211 (100.00%)	10,988,386 (100.00%)
Low quality reads	37,106 (0.27%)	9,399 (0.09%)
N% > 10% reads	722 (0.01%)	197 (0.00%)
Length <18nt and >30nt	3,576,896(26.37%)	3,162,932(28.78%)
5' adapter contamine	6,440 (0.05%)	7,265 (0.07%)
3' adapter null or Insert null	466,960 (3.44%)	329,058 (2.99%)
Clean reads	9,475,087(69.86%)	7,479,535(68.07%)
Unique reads	7,385,048 (54.45%)	6,653,378 (60.55%)

242 Table 2 summarizes the categories of unique reads. High levels of small RNA
243 expression from rRNA and NAT genes were observed in both libraries. The number
244 of miRNAs was more abundant in stem tissue as compared to the callus of tomato,
245 mainly due to the high expression of sly-miR171, sly-miR396 and sly-miR397.

246 **Table 2. Reads categories of two small RNA libraries derived from stem and**
247 **callus.**

Type	Number of reads (Percentage of reads)	
	Stem	Callus
Unique reads	7,385,048 (100%)	6,653,378 (100%)
Known miRNA	175634 (2.38%)	128569(1.93%)

rRNA	318166(4.31%)	295130(4.44%)
tRNA	0(0.00%)	0(0.00%)
snRNA	3493(0.05%)	4302(0.06%)
snoRNA	16964(0.23%)	18823(0.28%)
Repeat	525093(7.11%)	521017(7.83%)
NAT	648360(8.78%)	646505(9.72%)
Novel miRNA	28342(0.38%)	21589(0.33%)
TAS	16594(0.22%)	17806(0.27%)
Exon	203176(2.75%)	197359(2.96%)
Intron	357484(4.84%)	323339(4.86%)
Others ^a	5091742(68.95%)	4478939(67.32%)

248 ^aOthers, refers to the number and proportion of the sRNA aligned to the reference
249 sequences but not aligned to the known miRNA, ncRNA, repeat, NAT, novel miRNA,
250 TAS, Exon and Intron.

251 **Identification of known miRNAs**

252 To identify known miRNAs, sRNA sequences obtained from deep sequencing were
253 contrasted to other currently annotated miRNAs of known mature plant species in
254 miRBase. A total of 92 known miRNAs were identified, belonging to 29 miRNA
255 gene families in the two sRNA libraries. Overall, 88 and 91 mature miRNAs were
256 identified in the stem and callus tissues, respectively (S3 Table). As shown in Fig. 4,

257 the sly-miR159 family was the most abundantly expressed, while sly-miR9471,
258 sly-miR6022, sly-miR396, and sly-miR166 families were moderately abundant.
259 Furthermore, the secondary structures of known miRNAs are shown in Fig. 5 (A) and
260 S1 Fig.

261 **Fig. 4. Reads of known miRNA families at stem and callus.**

262 **Fig. 5. Secondary structure of identified miRNA precursors.**

263 The red protrusions are the mature sequences. (A) Known miRNA: sly-miR159; (B)
264 Novel miRNA: novel 110.

265 **Predicted novel miRNAs**

266 Unannotated miRNAs were used to predict novel miRNAs. We identified 91 novel
267 miRNAs were identified in total, of which 82 were mapped in both libraries (S4
268 Table). The expression levels of novel miRNAs were distinctly different. Most of
269 them showed comparatively low expression levels (63 novel miRNAs in stem
270 samples and 68 novel miRNAs in callus had less than 120 raw reads). In contrast, two
271 novel miRNAs (annotated as novel 1 and novel 9) in both libraries contained more
272 than 1,000 reads. The most abundantly expressed novel miRNA was novel 1 with a
273 total of 23,938 reads in both libraries. The predicted secondary structures of novel
274 pre-miRNAs are showed in Fig. 5 (B) and S2 Fig.

275 **Identification of differentially expressed miRNAs**

276 A total of 49 known and 44 novel miRNAs pertaining to the two libraries were
277 expressed with significant differences with regards to \log_2 (callus/stem) (>1 or <-1)
278 and P-value (<0.05) criteria (Fig. 6) (S5 Table). For known miRNAs, 24 miRNAs
279 were up-regulated and 25 miRNAs were down-regulated in callus vs. stem tissue
280 samples. Among the novel miRNAs, 17 were up-regulated and 27 were
281 down-regulated in callus vs. stem tissues (Fig. 7). When miRNA distributions were
282 assessed between the two libraries, 44 known defined miRNAs and 37 novel miRNAs
283 were generally expressed in both libraries. The comparison of miRNA expression
284 showed that 1 known and 7 novel miRNAs were expressed only in the stem, while 4
285 novel miRNAs were expressed solely in callus tissue, respectively (Fig. 8).

286 **Fig. 6. Cluster analyses of differentially expressed miRNAs.**

287 Red denotes highly expressed miRNAs, while blue denotes weakly expressed
288 miRNAs. The color is from red to blue, indicating that \log_{10} (TPM + 1) is from large
289 to small.

290 **Fig. 7. The number of known and novel up- and down- regulated miRNAs in** 291 **callus vs. stem tissue.**

292 **Fig. 8. Venn diagram of the number of specifically expressed miRNAs at stem** 293 **and callus.**

294 (A) Known miRNAs; (B) Novel miRNAs.

295 The miRNAs with lower p-value include sly-miR166 and sly-miR397. The
296 significantly down-regulated expression of sly-miR166 in callus cells could be related
297 to its role in promoting callus formation by down-regulating homeodomain leucine
298 zipper class III (HD-ZIP III) levels [49–51]. The most significantly down-regulated
299 gene in callus tissue was sly-miR397, which is known to play an important role in the
300 accumulation of laccases during callus formation[52,53].

301 To confirm miRNAs expression levels in stem and callus and verify the
302 deep-sequencing results, four known and two novel miRNAs were selected randomly
303 for qRT-PCR. These miRNAs expression patterns resembled the deep-sequencing
304 results, suggesting that sRNA sequencing data were reliable (Fig. 9).

305 **Fig. 9. The relative expression levels of 6 (four known and two novel) miRNAs by**
306 **qRT-PCR.**

307 The bars represents the relative expression and standard deviation of the 6 miRNAs.
308 qRT-PCR value of miRNAs in stem was set to 1, and values of miRNAs in callus
309 were scaled.

310 **Target prediction**

311 To analyze the biological functions of differentially expressed miRNAs in stem and
312 callus tissues, the psRobot software was used to predict target genes. Among 1186
313 predicted target genes, a total of 505 known and 6 novel differentially expressed
314 miRNA target genes were identified (S6 Table). Functional annotations of BLAST

315 analysis for predicted target genes indicated that these targets contained mRNA
316 coding regions for zinc finger protein (sly-miR165, sly-miR164, sly-miR391,
317 sly-miR394, sly-miR396, sly-miR477, sly-miR482, sly-miR6027, sly-miR9469,
318 sly-miR9478, and sly-miR9479), and MYB (sly-miR156, sly-miR319, sly-miR9469,
319 and sly-miR9478) protein. Furthermore, some miRNAs were found to target
320 transcription factors, such as SQUAMOSA promoter binding protein-like gene (SPL)
321 (sly-miR156)[54], Auxin response factors (ARFs) (sly-miR160)[55], HD-ZIP III
322 (sly-miR166)[56], NAM (sly-miR164 and sly-miR9478)[57], and MADS
323 (sly-miR396 and sly-miR9477)[58], which are all known to be involved in plant
324 regeneration. Laccase(sly-miR397) was also important in the regulation of plant
325 development and regeneration[22,23]. The target genes of some miRNAs specifically
326 expressed in the callus were CCAAT-binding (sly-miR169a), zinc finger
327 (sly-miR9469-3p and sly-miR9469-5p), SQUAMOSA promoter binding protein
328 (SBP-box), MADS-box and K-box (sly-miR9477-5p). Interestingly, all the target
329 genes of novel 46 (solyc05g015840.2, solyc12g038520.1, solyc10g078700.1,
330 solyc05g015510.2, solyc05g012040.2, and solyc04g045560.2) were the same as those
331 targeted by sly-miR156e-5p, sly-miR156d-5p, and sly-miR156a.

332 **Discussion**

333 Recently great progress has been made in understanding the role of miRNAs in
334 regulating the transitions between different development stage in plants, such as those

335 from vegetative-to-reproductive, juvenile-to-adult and aerial stem-to-rhizome
336 transitions[59–62]. In the present study, we demonstrate that miRNAs are involved in
337 complex regulatory networks during stem-callus transition during *in vivo* regeneration
338 of tomato. We identified a total of 183 miRNAs (92 conserved and 91 novel
339 miRNAs) by next-generation sequencing. Previous studies on miRNAs, including Xu
340 et al.[30] identified 289 known miRNAs and 1087 novel miRNAs in longan, while
341 Wu et al. [26] reported 50 known and 45 novel miRNAs in citrus. Taken together,
342 these data show that distinct types of miRNAs are expressed at different levels during
343 the process of regeneration in different species.

344 Cytokinin triggers a complex gene expression program in plant tissue culture that
345 results in adventitious shoot regeneration[63]. The current model for cytokinin signal
346 transduction is a multi-step phosphorelay. First, *Arabidopsis* histidine kinase (AHKs),
347 the cytokinin receptors in the plasma membrane, perceive the cytokinin signal
348 triggering a multi-step phosphorelay. At the end of this pathway, B-ARR receives the
349 phosphoryl group and becomes active. As transcription factors, B-type ARR can
350 activate the expression of cytokinin-responsive genes and A-type ARR. Interestingly,
351 the expression of A-type ARR interferes with the function of B-type ARR proteins
352 through a negative feedback loop[64,65]. Cytokinin thus plays a vital role during *in*
353 *vitro* regeneration. It can not only induce adventitious buds alone, but also cooperate
354 with auxin. Many studies have confirmed that miRNA regulate hormone signaling
355 genes involved in regeneration.

356 **MiR156/SPL module involved in callus formation by**
357 **regulating cytokinin signaling pathway**

358 Siddiqui et al. [66] summarized the most common expressed miRNAs during SEG in
359 11 economically plants. Of these, miRNA156 was found to be most frequently
360 detected in six of the 11 plant species tested. Sequences of miR156 were highly
361 conserved in plants [67]. In the present study, sly-miR156d-5p and sly-miR156e-5p
362 were newly identified and shown to be expressed differently in stem and callus tissue.
363 Promoter binding protein (SBP) domain SQUAMOSA was predicted to be one of the
364 targets of sly-miR156d-5p and sly-miR156e-5p. SBP domain proteins, putative
365 plant-specific transcription factor gene families, have been shown to participate in
366 various plant biological processes and to be involved in vegetative-to-reproductive
367 phase transition[68–70], pollen sac development[71], gibberellins (GAs) signaling
368 network [72] and establishment of lateral meristems[73]. As SBP-box gene family
369 members, 10 of 16 *SPL* genes were shown to be targets of miR156 in *Arabidopsis*,
370 while 10 of the 15 *SPL* genes were proposed to be targets of miR156 in citrus [74,75].
371 The function of the miR156-SPLs module was confirmed to be crucial in callus
372 production in citrus *in vitro* callus through targeted inhibition of miR156-targeted
373 SPLs and over-expression of csi-miR156a[20]. Therefore, differential expression
374 levels of miR156 during tomato callus generation in the present study, suggest that is
375 likely to play an important role in *in vivo* regeneration.
376 Zhang et al. [76] demonstrated that miR156 participates in regulation of shoot

377 regeneration *in vitro*. MiR156 expression gradually increases with age and suppresses
378 the expression of its target *SPL* genes. Down-regulated SPLs attenuate cytokinin
379 signaling by binding to the B-type Arabidopsis response regulators (ARR)
380 transcription factor. The data presented here show that cytokinin levels increase
381 during *in vivo* regeneration in tomato. However, sly-miR156d-5p and sly-miR156e-5p
382 were found to be up- and down-regulated, respectively. Thus, the regulation of
383 miR156-SPL-ARR module during *in vivo* callus formation and shoot regeneration in
384 tomato needs to be further investigated.

385 **IAA level regulated by miR166 in callus formation**

386 Low expression levels of sly-miR166c-5p and sly-miR166c-3p were observed during
387 the change from stem to callus stages in this study. Previous research has shown that
388 miR166, together with miR156 and miR396 were down-regulated during callus
389 formation from tea plant stem explants[77]. miR166 was identified to target Class III
390 homeodomain leucine zipper (HD-Zip III) gene family of transcription factors,
391 including REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV),
392 CORONA (CNA), and ATHB8 in *Arabidopsis* [49]. HD-ZIP III proteins play an
393 important role in plant regeneration by regulating the differentiation of stem cells and
394 the establishment of shoot apical meristem (SAM) and RAM [78–80].
395 More recently, REV was demonstrated to activate genes upstream of several auxin
396 biosynthesis, transport, and response genes. Brandt et al. [81] identified that REV

397 targeted the auxin biosynthetic enzymes TAA1 and YUCCA5(YUC5), and directly
398 affected the levels of free auxin. In *Arabidopsis*, loss-of-function mutants of REV
399 showed lower expression levels of the PIN1 and PIN2 auxin transporters and
400 reduction in the tip-to-base transport of auxin[82]. Additionally, REV function is
401 necessary for polar auxin transport in the shoot[83] . Li et al.[51,84,85] demonstrated
402 that over-expression of LaMIR166a and down-regulated of LaHDZIP31-34 genes
403 results in different IAA levels in pro-embryogenic masses of *L. leptolepis*. The
404 authors speculated that LaMIR166 targeted HD-ZIP III genes likely regulate auxin
405 biosynthesis and response genes. Overall, these results indicated the complex
406 regulatory relationships between miR166 and plant development. Further, Ma et al.
407 [62] reviewed five key microRNAs involved in developmental phase transitions in
408 seed plant, and miR166 was one of them.

409 **Other miRNAs related to the phytohormone signaling during**
410 **in vivo regeneration**

411 There is no doubt that auxin signaling and transport is a versatile trigger of plant
412 developmental changes including regeneration [86]. Based on a number of previous
413 studies, which focussed on miRNAs involved in regulation of the auxin signaling, in
414 *Arabidopsis*, miR393 was shown to contribute to SE, leaf development and
415 antibacterial resistance by repressing auxin signaling[87–89]. Two auxin response
416 factors genes, *ARF6* and *ARF8*, are targeted by miR167 [90]. During callus formation,

417 miR160 was defined as a key repressor by modulating the interplay between auxin
418 and cytokinin. The callus initiation was repressed by over-expression of miR160 or
419 reduced expression of its target *ARF10*. *ARF10* can inhibit cytokinin signaling A-type
420 genes *ARR15*[91]. Although A-type genes *ARR15* and *ARR7* were identified to inhibit
421 callus formation, those of the B-type genes *ARR1* and *ARR21* can enhance its
422 initiation[92,93](Fig. 10).

423 **Fig. 10. Genetic networks of callus formation during *in vivo* regeneration of**
424 **tomato regulated by miRNA-target modules together with their downstream**
425 **targets.**

426 Arrows represent activation, while lines with a bar represent repression. The solid
427 lines represent the results predicted by this study, and the dotted lines represent the
428 results from references. The up-regulated miRNAs are shown in the blue box, while
429 the down-regulated miRNAs are shown in orange ones. miRNA targets are shown in
430 green oval frames.

431
432 Cell proliferation relied not only on high levels of auxin but also on low level of
433 cytokinin during in vitro callus induction in *Arabidopsis* [94]. This study showed the
434 down-regulation of sly-miR160 and low concentrations of cytokinin in callus were
435 crucial in callus formation. We predict that a similar interplay between
436 microRNA/phytohormone levels may exist between *in vivo* and *in vitro* regeneration
437 in tomato.

438 **MiR397 repressed callus formation through inhibition of**
439 **laccases expression**

440 The expression of Sly-miR397 was most significantly down-regulated in callus tissue.
441 MiR397 has been validated to target laccases (*LAC2* and *LAC17* in this study), a
442 group of polyphenol oxidases[52,95]. In higher plants, laccases are associated with
443 lignin and xylem synthesis, and are proposed to play a role in secondary cell wall
444 thickening [23,96,97]. Lignin is an essential component of plant secondary cell walls,
445 which influence plant growth and differentiation[98]. Previous studies indicated that
446 callus tissue first contains lignified parenchyma cells, followed by the formation of
447 short vessels and traumatic resin ducts after plant injury, and the induction of vessels
448 requires the involvement of lignin[53,99]. Overall, we predict that low expression
449 levels of *Sly-miR397* in callus tissue permits the accumulation of laccases, leading to
450 the increase of lignin deposition within the callus.

451 **Conclusion**

452 We used a new model system to study the dynamic changes in trans-zeatin levels and
453 the regulatory patterns of miRNA expression during *in vivo* regeneration of tomato.
454 The significant changes in trans-zeatin levels at 0, 9, 12, 15, 18, 21, 24 and 30 d after
455 decapitation proves that trans-zeatin plays a crucial role during *in vivo* regeneration in
456 tomato. However, the treatment with excess lovastatin on the cut surface of tomato
457 stems did not inhibit callus formation, which indicated that *de novo* biosynthesis of

458 cytokinin did not occur in the cut surface of tomato stems. A total of 92 known and 91
459 novel miRNAs were identified from the stem explant and the callus regenerated from
460 the cutting surfaces after decapitation, respectively, of which 49 known and 44 novel
461 miRNAs exhibited differential expression between the two libraries. In addition, a
462 total of 505 known miRNA target genes and 6 novel miRNA target genes were further
463 identified. We predict that these differentially expressed miRNAs and their relevant
464 target genes play an important role in callus formation during *in vivo* regeneration of
465 tomato. Among these, sly-miR156, sly-miR160, sly-miR166, and sly-miR397 are
466 predicted to be involved in callus formation during *in vivo* regeneration of tomato by
467 targeting SPL, HD-ZIP III, ARFs, and LAC proteins, as well as by regulating
468 cytokinin, IAA, and laccase levels. The findings of this study provide a useful
469 resource for further investigation on callus formation during *in vivo* regeneration of
470 tomato.

471 **References**

472 1. Altpeter F, Springer NM, Bartley LE, Blechl A, Brutnell TP, Citovsky V, et al.
473 Advancing Crop Transformation in the Era of Genome Editing. *Plant Cell*. 2016;
474 tpc.00196.2016. doi:10.1105/tpc.16.00196

475 2. Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas DF. Plant
476 gene editing through de novo induction of meristems. *Nat Biotechnol*. 2020;38: 84–
477 89. doi:10.1038/s41587-019-0337-2

478 3. Zuo J, Niu Q-W, Frugis G, Chua N-H. The WUSCHEL gene promotes
479 vegetative-to-embryonic transition in *Arabidopsis*. *Plant J.* 2002;30: 349–359.
480 doi:10.1046/j.1365-313x.2002.01289.x

481 4. Barton MK. Twenty years on: The inner workings of the shoot apical meristem, a
482 developmental dynamo. *Developmental Biology.* 2010;341: 95–113.
483 doi:10.1016/j.ydbio.2009.11.029

484 5. Lowe K, Wu E, Wang N, Hoerster G, Hastings C, Cho M-J, et al. Morphogenic
485 Regulators Baby boom and Wuschel Improve Monocot Transformation. *Plant Cell.*
486 2016;28: 1998–2015. doi:10.1105/tpc.16.00124

487 6. Nelson-Vasilchik K, Hague J, Mookan M, Zhang ZJ, Kausch A. Transformation
488 of Recalcitrant Sorghum Varieties Facilitated by Baby Boom and Wuschel2. *Curr
489 Protoc Plant Biol.* 2018;3: e20076. doi:10.1002/cppb.20076

490 7. Yin L, Lan Y, Zhu L. Analysis of the protein expression profiling during rice
491 callus differentiation under different plant hormone conditions. *Plant Mol Biol.*
492 2008;68: 597–617. doi:10.1007/s11103-008-9395-4

493 8. Rodrigues AS, Chaves I, Costa BV, Lin Y-C, Lopes S, Milhinhos A, et al. Small
494 RNA profiling in *Pinus pinaster* reveals the transcriptome of developing seeds and
495 highlights differences between zygotic and somatic embryos. *Sci Rep.* 2019;9: 11327.
496 doi:10.1038/s41598-019-47789-y

497 9. Amutha S, Kathiravan K, Singer S, Jashi L, Shomer I, Gaba SV. Adventitious
498 Shoot Formation in Decapitated Dicotyledonous Seedlings Starts with Regeneration

499 of Abnormal Leaves from Cells Not Located in a Shoot Apical Meristem. *Vitro*
500 *Cellular & Developmental Biology Plant*. 2009;45: 758–768. doi:10.2307/25623037

501 10. Masafumi J, Genjirou Mori, Kazuhiko Mitsukuri. *In Vivo Shoot Regeneration*
502 *Promoted by Shading the Cut Surface of the Stem in Tomato Plants*. *Hortscience A*
503 *Publication of the American Society for Horticultural Science*. 2008;43: 220–222.
504 doi:10.21273/HORTSCI.43.1.220

505 11. Nielsen MD, Farestveit B, Andersen AS. *Adventitious Shoot Development from*
506 *Decapitated Plants of Periclinal Chimeric Poinsettia Plants (Euphorbia pulcherrima*
507 *Willd ex Klotsch)*. *European Journal of Horticultural Science*. 2003 [cited 12 Apr
508 2020]. Available: <http://www.jstor.org/stable/24126204>

509 12. Jones-Rhoades MW, Bartel DP, Bartel B. *MicroRNAs AND THEIR*
510 *REGULATORY ROLES IN PLANTS*. *Annual Review of Plant Biology*. 2006;57:
511 19–53. doi:10.1146/annurev.arplant.57.032905.105218

512 13. Man W, Fei Y, Wei W, Yu W, Han D, Qian L. *Epstein-Barr virus-encoded*
513 *microRNAs as regulators in host immune responses*. *International Journal of*
514 *Biological Sciences*. 2018;14: 565–576. doi:10.7150/ijbs.24562

515 14. Lima JCD, Loss-Morais G, Margis R. *MicroRNAs play critical roles during plant*
516 *development and in response to abiotic stresses*. *Genetics & Molecular Biology*.
517 2012;35: 1069–1077. doi:10.1590/S1415-47572012000600023

518 15. Li C, Zhang B. *MicroRNAs in Control of Plant Development*. *J Cell Physiol*.
519 2016;231: 303–313. doi:10.1002/jcp.25125

520 16. Smoczynska A, Szwejkowska-Kulinska Z. MicroRNA-mediated regulation of
521 flower development in grasses. *Acta Biochim Pol.* 2016;63: 687–692.
522 doi:10.18388/abp.2016_1358

523 17. Singh A, Gautam V, Singh S, Sarkar Das S, Verma S, Mishra V, et al. Plant small
524 RNAs: advancement in the understanding of biogenesis and role in plant
525 development. *Planta.* 2018;248: 545–558. doi:10.1007/s00425-018-2927-5

526 18. Chávez-Hernández EC, Alejandri-Ramírez ND, Juárez-González VT, Dinkova
527 TD. Maize miRNA and target regulation in response to hormone depletion and light
528 exposure during somatic embryogenesis. *Front Plant Sci.* 2015;6: 555.
529 doi:10.3389/fpls.2015.00555

530 19. Su YH, Liu YB, Zhou C, Li XM, Zhang XS. The microRNA167 controls somatic
531 embryogenesis in *Arabidopsis* through regulating its target genes ARF6 and ARF8.
532 *Plant Cell Tiss Organ Cult.* 2016;124: 405–417. doi:10.1007/s11240-015-0903-3

533 20. Long JM, Liu CY, Feng MQ, Yun L, Wu XM, Guo WW. miR156-SPL modules
534 regulate induction of somatic embryogenesis in citrus callus. *Journal of Experimental
535 Botany.* 2018; 12. doi:10.1093/jxb/ery132

536 21. Chu Z, Chen J, Xu H, Dong Z, Chen F, Cui D. Identification and Comparative
537 Analysis of microRNA in Wheat (*Triticum aestivum* L.) Callus Derived from Mature
538 and Immature Embryos during In vitro Culture. *Frontiers in Plant Science.* 2016;7.
539 doi:10.3389/fpls.2016.01302

540 22. Luo Y-C, Zhou H, Li Y, Chen J-Y, Yang J-H, Chen Y-Q, et al. Rice embryogenic

541 calli express a unique set of microRNAs, suggesting regulatory roles of microRNAs
542 in plant post-embryogenic development. *FEBS Letters.* 2006;580: 5111–5116.
543 doi:10.1016/j.febslet.2006.08.046

544 23. Chen CJ, liu Q, Zhang Y-C, Qu L-H, Chen Y-Q, Gautheret D. Genome-wide
545 discovery and analysis of microRNAs and other small RNAs from rice embryogenic
546 callus. *Rna Biology.* 2011;8: 538–547. doi:10.4161/rna.8.3.15199

547 24. Yang X, Wang L, Yuan D, Lindsey K, Zhang X. Small RNA and degradome
548 sequencing reveal complex miRNA regulation during cotton somatic embryogenesis.
549 *Journal of Experimental Botany.* 2013;64: 1521–1536. doi:10.1093/jxb/ert013

550 25. Gao C, Wang P, Zhao S, Zhao C, Xia H, Hou L, et al. Small RNA profiling and
551 degradome analysis reveal regulation of microRNA in peanut embryogenesis and
552 early pod development. *Bmc Genomics.* 2017;18: 220.
553 doi:10.1186/s12864-017-3587-8

554 26. Wu XM, Kou SJ, Liu YL, Fang YN, Xu Q, Guo WW. Genomewide analysis of
555 small RNAs in nonembryogenic and embryogenic tissues of citrus: microRNA- and
556 siRNA-mediated transcript cleavage involved in somatic embryogenesis. *Plant
557 Biotechnology Journal.* 2015;13: 383–394. doi:10.1111/pbi.12317

558 27. Sabana AA, Rajesh MK, Antony G. Dynamic changes in the expression pattern
559 of miRNAs and associated target genes during coconut somatic embryogenesis.
560 *Planta.* 2020;251: 79. doi:10.1007/s00425-020-03368-4

561 28. Zhang J, Zhang S, Han S, Wu T, Li X, Li W, et al. Genome-wide identification of

562 microRNAs in larch and stage-specific modulation of 11 conserved microRNAs and
563 their targets during somatic embryogenesis. *Planta.* 2012;236: 647–657.
564 doi:10.1007/s00425-012-1643-9

565 29. Yakovlev IA, Fossdal CG. In Silico Analysis of Small RNAs Suggest Roles for
566 Novel and Conserved miRNAs in the Formation of Epigenetic Memory in Somatic
567 Embryos of Norway Spruce. *Front Physiol.* 2017;8: 674.
568 doi:10.3389/fphys.2017.00674

569 30. Xu X, Chen X, Chen Y, Zhang Q, Su L, Chen X, et al. Genome-wide
570 identification of miRNAs and their targets during early somatic embryogenesis in
571 *Dimocarpus longan* Lour. *Sci Rep.* 2020;10: 4626. doi:10.1038/s41598-020-60946-y

572 31. Li T, Chen J, Qiu S, Zhang Y, Wang P, Yang L, et al. Deep sequencing and
573 microarray hybridization identify conserved and species-specific microRNAs during
574 somatic embryogenesis in hybrid yellow poplar. *PLoS ONE.* 2012;7: e43451.
575 doi:10.1371/journal.pone.0043451

576 32. Zhai L, Xu L, Wang Y, Huang D, Yu R, Limera C, et al. Genome-Wide
577 Identification of Embryogenesis-Associated microRNAs in Radish (*Raphanus*
578 *sativus*L.) by High-Throughput Sequencing. *Plant Molecular Biology Reporter.*
579 2014;32: 900–915. doi:10.1007/s11105-014-0700-x

580 33. Zhang J, Xue B, Gai M, Song S, Jia N, Sun H. Small RNA and Transcriptome
581 Sequencing Reveal a Potential miRNA-Mediated Interaction Network That Functions
582 during Somatic Embryogenesis in *Lilium pumilum* DC. *Fisch. Frontiers in Plant*

583 Science. 2017;8: 566-. doi:10.3389/fpls.2017.00566

584 34. Alejandri-Ramírez ND, Chávez-Hernández EC, Contreras-Guerra JL, Reyes JL,

585 Dinkova TD. Small RNA differential expression and regulation in Tuxpeño maize

586 embryogenic callus induction and establishment. Plant Physiol Biochem. 2018;122:

587 78–89. doi:10.1016/j.plaphy.2017.11.013

588 35. Zhou L, Chen J, Li Z, Li X, Hu X, Huang Y, et al. Integrated profiling of

589 microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell

590 renal cell carcinoma. PLoS ONE. 2010;5: e15224. doi:10.1371/journal.pone.0015224

591 36. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time

592 quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Research.

593 2005;33: e179. doi:10.1093/nar/gni178

594 37. Tezuka T, Harada M, Johkan M, Yamasaki S, Oda M. Effects of Auxin and

595 Cytokinin on In Vivo Adventitious Shoot Regeneration from Decapitated Tomato

596 Plants. Hortscience A Publication of the American Society for Horticultural Science.

597 2011;46: 1661–1665. doi:10.21273/HORTSCI.46.12.1661

598 38. Plastira VA, Perdikaris AK. EFFECT OF GENOTYPE AND EXPLANT TYPE

599 IN REGENERATION FREQUENCY OF TOMATO IN VITRO. Acta Horticulturae.

600 1997; 231–234. doi:10.17660/ActaHortic.1997.447.47

601 39. Sun H-J, Uchii S, Watanabe S, Ezura H. A highly efficient transformation

602 protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell

603 Physiol. 2006;47: 426–431. doi:10.1093/pcp/pcp251

604 40. Ehlert B, Schöttler MA, Tischendorf G, Ludwig-Müller J, Bock R. The
605 paramutated SULFUREA locus of tomato is involved in auxin biosynthesis. *J Exp
606 Bot.* 2008;59: 3635–3647. doi:10.1093/jxb/ern213

607 41. Park J, Lee Y, Martinoia E, Geisler M. Plant hormone transporters: what we
608 know and what we would like to know. *Bmc Biology.* 2017;15: 93.
609 doi:10.1186/s12915-017-0443-x

610 42. Hartig K, Beck E. Assessment of lovastatin application as tool in probing
611 cytokinin-mediated cell cycle regulation. *Physiologia Plantarum.* 2005;125: 260–267.
612 doi:10.1111/j.1399-3054.2005.00556.x

613 43. Crowell DN, Salaz MS. Inhibition of Growth of Cultured Tobacco Cells at Low
614 Concentrations of Lovastatin Is Reversed by Cytokinin. *Plant Physiology.* 1992;100:
615 2090–2095. doi:10.1104/pp.100.4.2090

616 44. Morris SE, Turnbull CG, Murfet IC, Beveridge CA. Mutational analysis of
617 branching in pea. Evidence that Rms1 and Rms5 regulate the same novel signal. *Plant
618 Physiol.* 2001;126: 1205–1213. doi:10.1104/pp.126.3.1205

619 45. Kuroha T, Kato H, Asami T, Yoshida S, Kamada H, Satoh S. A trans-zeatin
620 riboside in root xylem sap negatively regulates adventitious root formation on
621 cucumber hypocotyls. *J Exp Bot.* 2002;53: 2193–2200. doi:10.1093/jxb/erf077

622 46. Kudoyarova GR, Vysotskaya LB, Cherkkozyanova A, Dodd IC. Effect of partial
623 rootzone drying on the concentration of zeatin-type cytokinins in tomato (*Solanum
624 lycopersicum* L.) xylem sap and leaves. *J Exp Bot.* 2007;58: 161–168.

625 doi:10.1093/jxb/erl116

626 47. Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H.

627 Regulation of cytokinin biosynthesis, compartmentalization and translocation. *J Exp*

628 *Bot.* 2008;59: 75–83. doi:10.1093/jxb/erm157

629 48. Takei K, Yamaya T, Sakakibara H. *Arabidopsis CYP735A1 and CYP735A2*

630 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin. *J Biol*

631 *Chem.* 2004;279: 41866–41872. doi:10.1074/jbc.M406337200

632 49. Williams L, Grigg SP, Xie M, Christensen S, Fletcher JC. Regulation of

633 *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA

634 miR166g and its AtHD-ZIP target genes. *Development.* 2005;132: 3657–3668.

635 doi:10.1242/dev.01942

636 50. Boualem A, Laporte P, Jovanovic M, Laffont C, Plet J, Combier J-P, et al.

637 MicroRNA166 controls root and nodule development in *Medicago truncatula*. *Plant J.*

638 2008;54: 876–887. doi:10.1111/j.1365-313X.2008.03448.x

639 51. Li Z-X, Li S-G, Zhang L-F, Han S, Li W-F, Xu H, et al. Over-expression of

640 miR166a inhibits cotyledon formation in somatic embryos and promotes lateral root

641 development in seedlings of *Larix leptolepis*. *Plant Cell, Tissue and Organ Culture*

642 (PCTOC). 2016;127: 1–13. doi:10.1007/s11240-016-1071-9

643 52. Jones-Rhoades MW, Bartel DP. Computational Identification of Plant

644 MicroRNAs and Their Targets, Including a Stress-Induced miRNA. *Molecular Cell.*

645 2004;14: 787–799. doi:10.1016/j.molcel.2004.05.027

646 53. Oda Y, Mimura T, Hasezawa S. Regulation of secondary cell wall development
647 by cortical microtubules during tracheary element differentiation in *Arabidopsis* cell
648 suspensions. *Plant Physiol.* 2005;137: 1027–1036. doi:10.1104/pp.104.052613

649 54. Nagasaki H, Jun-ichi Ltoh, Hayashi K, Hibara KI, Satoh-Nagasaki N, Nosaka
650 M, et al. The small interfering RNA production pathway is required for shoot
651 meristem initiation in rice. *Proceedings of the National Academy of Sciences of the*
652 *United States of America.* 2007;104: p.14867-14871. doi:10.1073/pnas.0704339104

653 55. Dinkova TD. Maize miRNA and target regulation in response to hormone
654 depletion and light exposure during somatic embryogenesis. *Frontiers in Plant*
655 *Science.* 2015;6: 14.

656 56. Chu Z, Chen Junying, Xu Haixia, Dong Zhongdong, Chen Feng, Cui Dangqun.
657 Identification and Comparative Analysis of microRNA in Wheat (*Triticum aestivum*
658 L.) Callus Derived from Mature and Immature Embryos during In vitro Culture.
659 *Frontiers in Plant Science.* 2016;7. doi:10.3389/fpls.2016.01302

660 57. Ge F, Luo X, Huang X, Zhang Y, He X, Liu M, et al. Genome-wide analysis of
661 transcription factors involved in maize embryonic callus formation. *Physiol*
662 *Plantarum.* 2016;158: 452–462. doi:10.1111/ppl.12470

663 58. Prakash AP, Kumar PP. PkMADS1 is a novel MADS box gene regulating
664 adventitious shoot induction and vegetative shoot development in *Paulownia*
665 *kawakamii.* *Plant Journal.* 2002;29: 141–151. doi:10.1046/j.0960-7412.2001.01206.x

666 59. Tanaka N, Itoh H, Sentoku N, Kojima M, Sakakibara H, Izawa T, et al. The

667 COP1 Ortholog PPS Regulates the Juvenile-Adult and Vegetative-Reproductive
668 Phase Changes in Rice. *The Plant Cell*. 2011;23: 2143–2154.

669 60. Tian F, Li X, Wu Y, Xia K, Jie O, Zhang M, et al. Rice osa-miR171c Mediates
670 Phase Change from Vegetative to Reproductive Development and Shoot Apical
671 Meristem Maintenance by Repressing Four OsHAM Transcription Factors. *Plos One*.
672 2015;10: e0125833-. doi:10.1371/journal.pone.0125833

673 61. Yang Q, Liu S, Han X, Ma J, Deng W, Wang X, et al. Integrated transcriptome
674 and miRNA analysis uncovers molecular regulators of aerial stem-to-rhizome
675 transition in the medical herb *Gynostemma pentaphyllum*. *BMC Genomics*. 2019;20:
676 865. doi:10.1186/s12864-019-6250-8

677 62. Ma J, Zhao P, Liu S, Yang Q, Guo H. The Control of Developmental Phase
678 Transitions by microRNAs and Their Targets in Seed Plants. *Int J Mol Sci*. 2020;21.
679 doi:10.3390/ijms21061971

680 63. Howell SH, Lall S, Che P. Cytokinins and shoot development. *Trends in Plant
681 Science*. 2003;8: 0–459. doi:10.1016/s1360-1385(03)00191-2

682 64. Kieber JJ, Ferreira FJ. Cytokinin signaling. *Current Opinion in Plant Biology*.
683 2005;8: 518–525. doi:10.1016/j.pbi.2005.07.013

684 65. Hwang I, Sheen J, Müller B. Cytokinin signaling networks. *Annu Rev Plant Biol*.
685 2012;63: 353–380. doi:10.1146/annurev-arplant-042811-105503

686 66. Siddiqui ZH, Abbas ZK, Ansari MW, Khan MN. The role of miRNA in somatic
687 embryogenesis. *Genomics*. 2019;111: 1026–1033. doi:10.1016/j.ygeno.2018.11.022

688 67. Madhuri Gandikota, Rainer P. Birkenbihl, Susanne Höhmann. The
689 miRNA156/157 recognition element in the 3' UTR of the *Arabidopsis* SBP box gene
690 SPL3 prevents early flowering by translational inhibition in seedlings. *Plant Journal*.
691 2007 [cited 3 Apr 2020]. doi:10.1111/j.1365-313X.2006.02983.x

692 68. Cardon GH, Höhmann S, Nettesheim K, Saedler H, Huijser P. Functional
693 analysis of the *Arabidopsis thaliana* SBP-box gene SPL3: A novel gene involved in
694 the floral transition. *Plant Journal*. 1997;12: 367–377.
695 doi:10.1046/j.1365-313X.1997.12020367.x

696 69. Wu G, Poethig RS. Temporal regulation of shoot development in *Arabidopsis*
697 *thaliana* by miR156 and its target SPL3. *Development*. 2006;133: 3539–3547.
698 doi:10.1242/dev.02521

699 70. Wang JW, Schwab R, Czech B, Mica E, Weigel D. Dual Effects of
700 miR156-Targeted SPL Genes and CYP78A5/KLUH on Plastochron Length and
701 Organ Size in *Arabidopsis thaliana*. *Plant Cell*. 2008;20: 1231–1243.
702 doi:10.1105/tpc.108.058180

703 71. Unte U, Sorensen A-M, Pesaresi P, Gandikota M, Leister D, Saedler H, et al.
704 SPL8, an SBP-Box Gene That Affects Pollen Sac Development in *Arabidopsis*. *The
705 Plant cell*. 2003;15: 1009–19. doi:10.1105/tpc.010678

706 72. Zhang Y, Schwarz S, Saedler H, Huijser P. SPL8, a local regulator in a subset of
707 gibberellin-mediated developmental processes in *Arabidopsis*. *Plant Molecular
708 Biology*. 2007;63: 429–439. doi:10.1007/s11103-006-9099-6

709 73. Chuck G, Whipple C, Jackson D, Hake S. The maize SBP-box transcription
710 factor encoded by *tasselsheath4* regulates bract development and the establishment of
711 meristem boundaries. *Development*. 2010;137: 1243–1250. doi:10.1242/dev.048348

712 74. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction
713 of Plant MicroRNA Targets. *Cell*. 2002;110: 0–520.
714 doi:10.1016/s0092-8674(02)00863-2

715 75. Liu M, Wu X, Long J, Guo W-W. Genomic characterization of miR156 and
716 *SQUAMOSA* promoter binding protein-like genes in sweet orange (*Citrus sinensis*).
717 *Plant Cell, Tissue and Organ Culture (PCTOC)*. 2017;130: 1–14.
718 doi:10.1007/s11240-017-1207-6

719 76. Zhang T-Q, Lian H, Tang H, Dolezal K, Zhou C-M, Yu S, et al. An intrinsic
720 microRNA timer regulates progressive decline in shoot regenerative capacity in
721 plants. *Plant Cell*. 2015;27: 349–360. doi:10.1105/tpc.114.135186

722 77. Gao Y, Li D, Zhang L-L, Borthakur D, Li Q-S, Ye J-H, et al. MicroRNAs and
723 their targeted genes associated with phase changes of stem explants during tissue
724 culture of tea plant. *Scientific Reports*. 2019;9: 20239.
725 doi:10.1038/s41598-019-56686-3

726 78. Jung J-H, Park C-M. MIR166/165 genes exhibit dynamic expression patterns in
727 regulating shoot apical meristem and floral development in *Arabidopsis*. *Planta*.
728 2007;225: 1327–1338. doi:10.1007/s00425-006-0439-1

729 79. Wong CE, Zhao Ying-Tao, Wang Xiu-Jie, Larry C, Wang Zhong-Hua, Farzad H,

730 et al. MicroRNAs in the shoot apical meristem of soybean. *Journal of Experimental*
731 *Botany*. 2011; 8. doi:10.1093/jxb/erq437

732 80. Turchi L, Baima S, Morelli G, Ruberti I. Interplay of HD-Zip II and III
733 transcription factors in auxin-regulated plant development. *J Exp Bot*. 2015;66: 5043–
734 5053. doi:10.1093/jxb/erv174

735 81. Brandt R, Salla-Martret M, Bou-Torrent J, Musielak T, Stahl M, Lanz C, et al.
736 Genome-wide binding-site analysis of REVOLUTA reveals a link between leaf
737 patterning and light-mediated growth responses. *Plant J*. 2012;72: 31–42.
738 doi:10.1111/j.1365-313X.2012.05049.x

739 82. Zhong R, Ye ZH. Alteration of auxin polar transport in the *Arabidopsis ifl1*
740 mutants. *Plant Physiol*. 2001;126: 549–563. doi:10.1104/pp.126.2.549

741 83. Huang T, Harrar Y, Lin C, Reinhart B, Newell NR, Talavera-Rauh F, et al.
742 *Arabidopsis KANADI1* acts as a transcriptional repressor by interacting with a
743 specific cis-element and regulates auxin biosynthesis, transport, and signaling in
744 opposition to HD-ZIP III factors. *Plant Cell*. 2014;26: 246–262.
745 doi:10.1105/tpc.113.111526

746 84. Li ZX, Zhang LF, Li WF, Qi LW, Han SY. MIR166a Affects the Germination of
747 Somatic Embryos in *Larixleptolepis* by Modulating IAA Biosynthesis and Signaling
748 Genes. *Journal of Plant Growth Regulation*. 2017 [cited 1 May 2020].
749 doi:10.1007/s00344-017-9693-7

750 85. Li Z-X, Fan Y-R, Dang S-F, Li W-F, Qi L-W, Han S-Y. LaMIR166a-mediated

751 auxin biosynthesis and signalling affect somatic embryogenesis in *Larix leptolepis*.

752 *Mol Genet Genomics*. 2018;293: 1355–1363. doi:10.1007/s00438-018-1465-y

753 86. Vanneste S, Friml J. Auxin: a trigger for change in plant development. *Cell*.

754 2009;136: 1005–1016. doi:10.1016/j.cell.2009.03.001

755 87. Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, et al. A plant

756 miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science*.

757 2006;312: 436–439. doi:10.1126/science.1126088

758 88. Si-Ammour A, Windels D, Arn-Bouloires E, Kutter C, Ailhas J, Meins F, et al.

759 miR393 and secondary siRNAs regulate expression of the TIR1/AFB2 auxin receptor

760 clade and auxin-related development of *Arabidopsis* leaves. *Plant Physiol*. 2011;157:

761 683–691. doi:10.1104/pp.111.180083

762 89. Wójcik AM, Gaj MD. miR393 contributes to the embryogenic transition induced

763 in vitro in *Arabidopsis* via the modification of the tissue sensitivity to auxin treatment.

764 *Planta*. 2016;244: 231–243. doi:10.1007/s00425-016-2505-7

765 90. Wu M-F, Tian Q, Reed JW. *Arabidopsis* microRNA167 controls patterns of

766 ARF6 and ARF8 expression, and regulates both female and male reproduction.

767 *Development*. 2006;133: 4211–4218. doi:10.1242/dev.02602

768 91. Liu Z, Li J, Wang L, Li Q, Lu Q, Yu Y, et al. Repression of callus initiation by

769 the miRNA-directed interaction of auxin-cytokinin in *Arabidopsis thaliana*. *Plant*

770 *Journal*. 2016 [cited 15 Mar 2020]. doi:10.1111/tpj.13211

771 92. Sakai H, Honma T, Aoyama T, Sato S, Kato T, Tabata S, et al. ARR1, a

772 transcription factor for genes immediately responsive to cytokinins. *Science*.
773 2001;294: 1519–1521. doi:10.1126/science.1065201

774 93. Buechel S, Leibfried A, To JPC, Zhao Z, Andersen SU, Kieber JJ, et al. Role of
775 A-type ARABIDOPSIS RESPONSE REGULATORS in meristem maintenance and
776 regeneration. *Eur J Cell Biol*. 2010;89: 279–284. doi:10.1016/j.ejcb.2009.11.016

777 94. Gordon SP, Heisler MG, Reddy GV, Ohno C, Das P, Meyerowitz EM. Pattern
778 formation during de novo assembly of the Arabidopsis shoot meristem. *Development*.
779 2007;134: 3539–3548. doi:10.1242/dev.010298

780 95. Sunkar R, Zhu J-K. Novel and stress-regulated microRNAs and other small
781 RNAs from Arabidopsis. *Plant Cell* 16, 2001–2019. *Plant Cell*. 2004;16: 2001–2019.
782 doi:10.1105/tpc.104.022830

783 96. Constabel CP, Yip L, Patton JJ, Christopher ME. Polyphenol oxidase from hybrid
784 poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol*.
785 2000;124: 285–295. doi:10.1104/pp.124.1.285

786 97. Lu S, Li Q, Wei H, Chang M-J, Tunlaya-Anukit S, Kim H, et al. Ptr-miR397a is a
787 negative regulator of laccase genes affecting lignin content in *Populus trichocarpa*.
788 *Proc Natl Acad Sci USA*. 2013;110: 10848–10853. doi:10.1073/pnas.1308936110

789 98. Dauwe R, Morreel K, Goeminne G, Gielen B, Rohde A, Van Beeumen J, et al.
790 Molecular phenotyping of lignin-modified tobacco reveals associated changes in
791 cell-wall metabolism, primary metabolism, stress metabolism and photorespiration.
792 *Plant J*. 2007;52: 263–285. doi:10.1111/j.1365-313X.2007.03233.x

793 99. Motose H, Fukuda H, Sugiyama M. Involvement of local intercellular
794 communication in the differentiation of zinnia mesophyll cells into tracheary
795 elements. *Planta*. 2001;213: 121–131. doi:10.1007/s004250000482

797 **Supporting information captions**

798 **S1 Fig. The secondary structures of known miRNAs.** The whole sequences are
799 miRNA precursors, and the red prominent parts are the mature sequences.

800 **S2 Fig. The secondary structures of novel pre-miRNAs.** The whole sequences are
801 miRNA precursors, and the red prominent parts are the mature sequences.

802 **S1 Table. Primers used in this study for qRT-PCR.**

803 **S2 Table. Quality of raw reads of two sRNA libraries produced from stem and**
804 **callus.**

805 ^a Q20, The percentage of bases with Phred value greater than 20 in the total bases.

806 ^b Q30, The percentage of bases with Phred value greater than 30 in the total bases

807 **S3 Table. Detailed information of known miRNAs in two small RNA libraries**
808 **derived from stem and callus.**

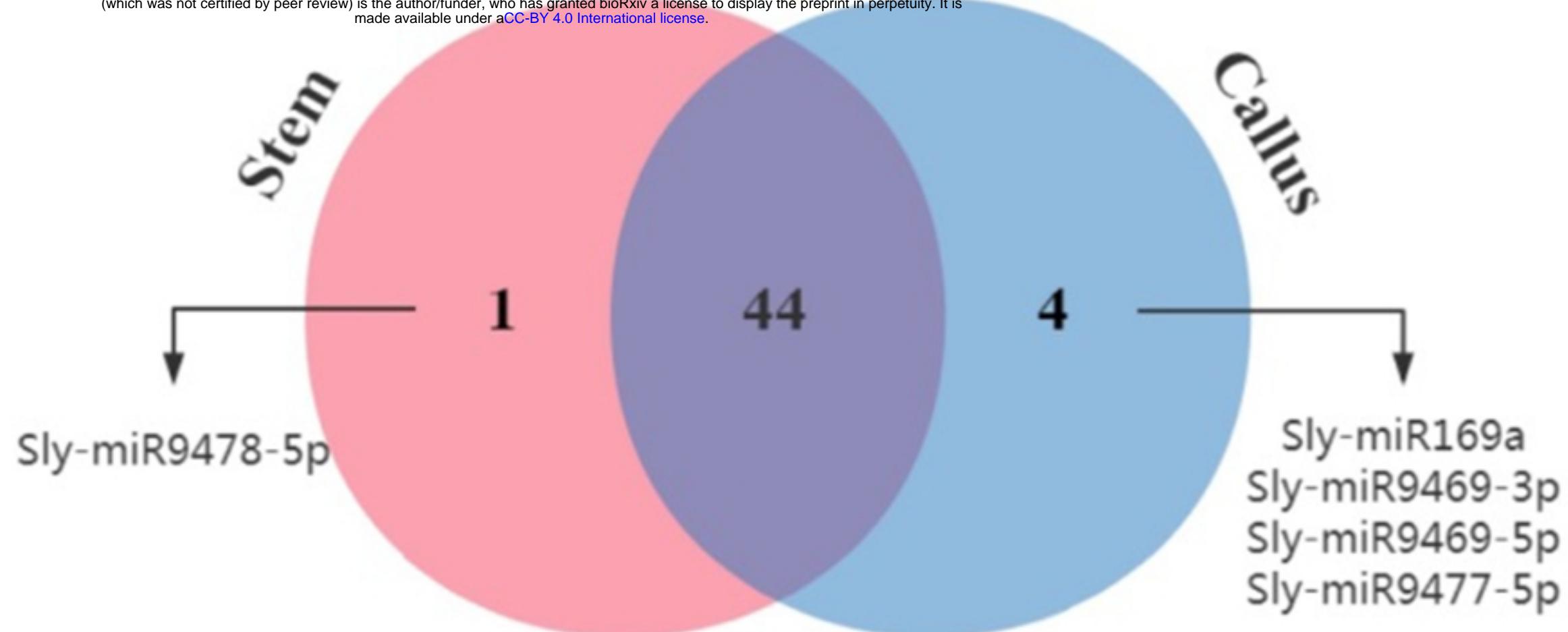
809 **S4 Table. Novel miRNAs of two small RNA libraries derived from stem and**
810 **callus.**

811 **S5 Table. Differentially expressed known and novel miRNAs during callus**
812 **formation of tomato *in vivo* regeneration.**

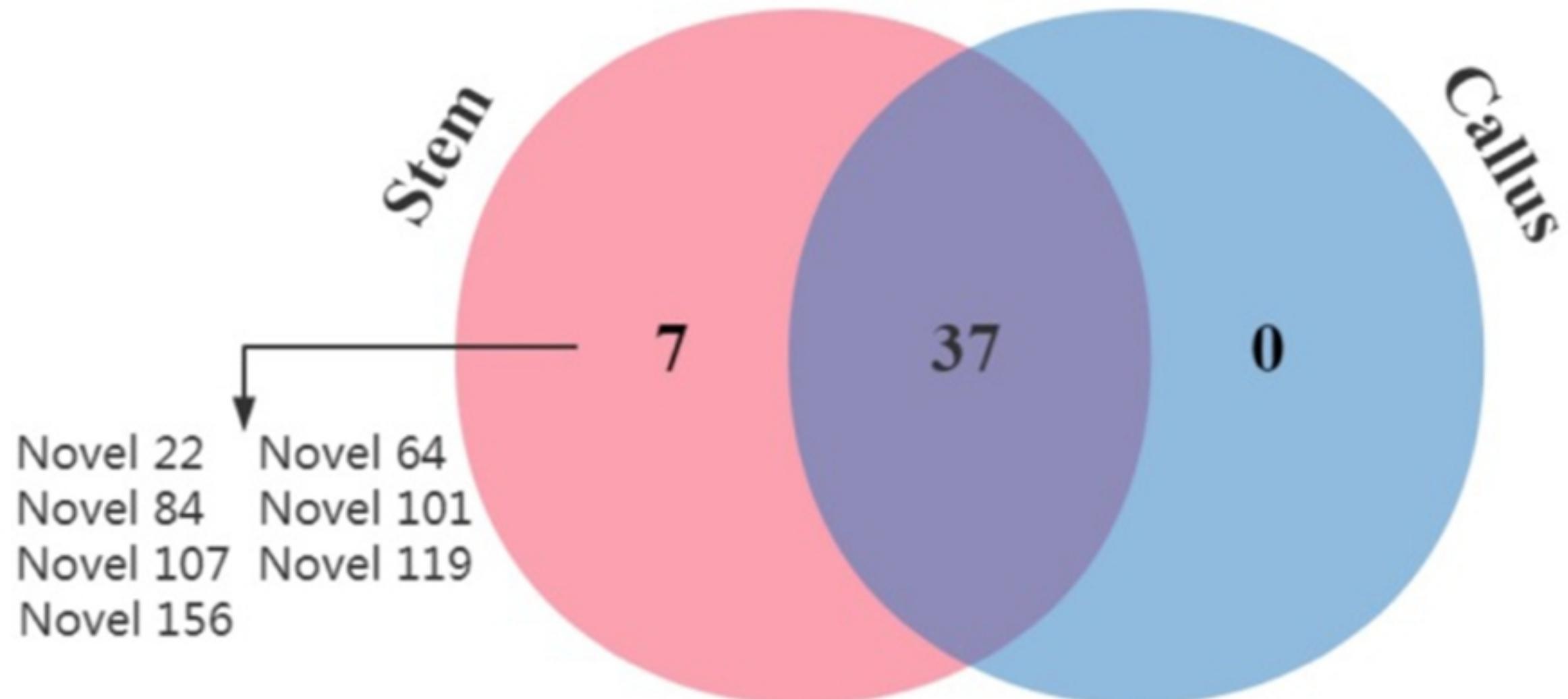
813 **S6 Table. Target genes for differentially expressed known and novel miRNAs.**

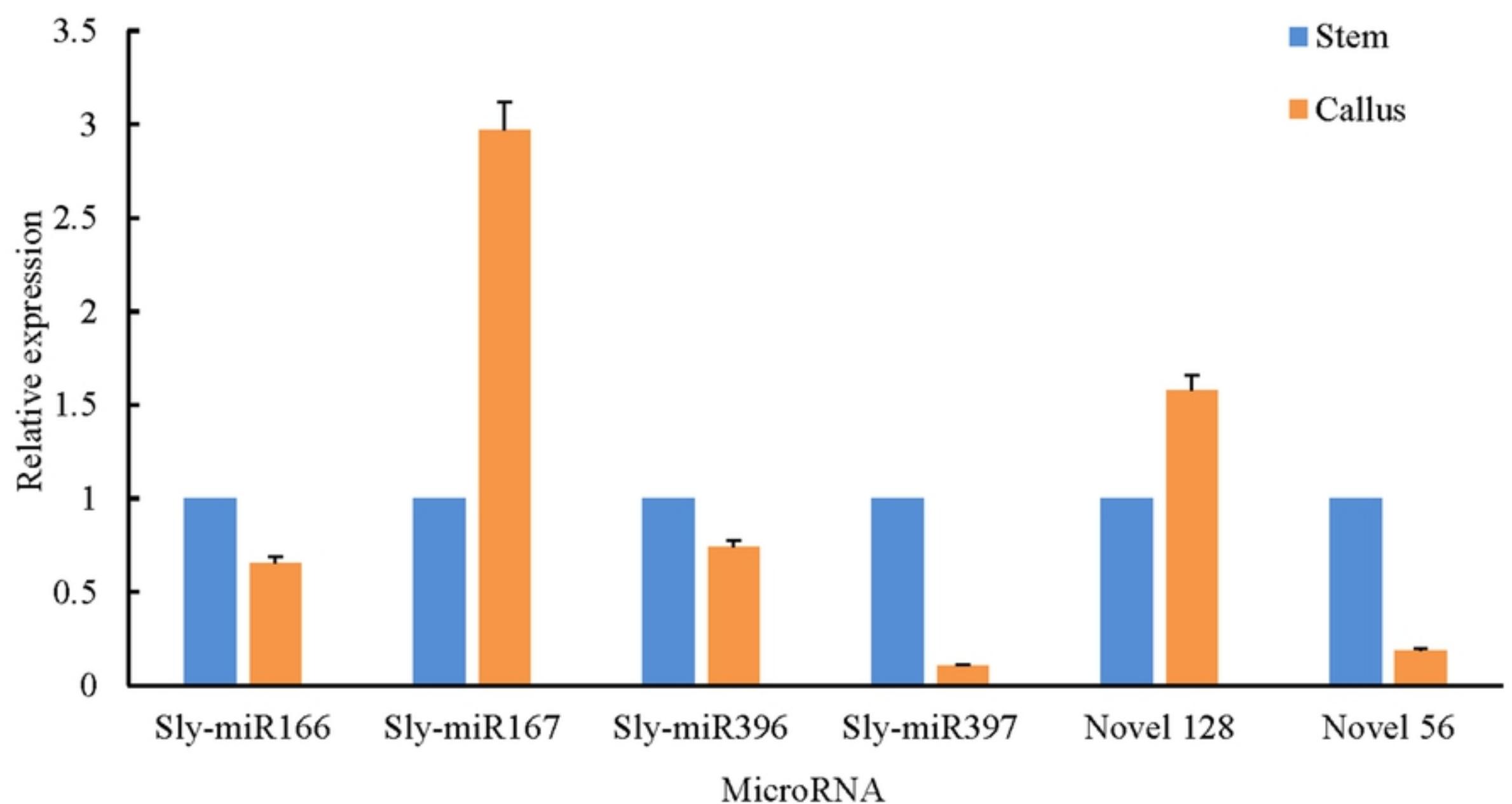
(A)

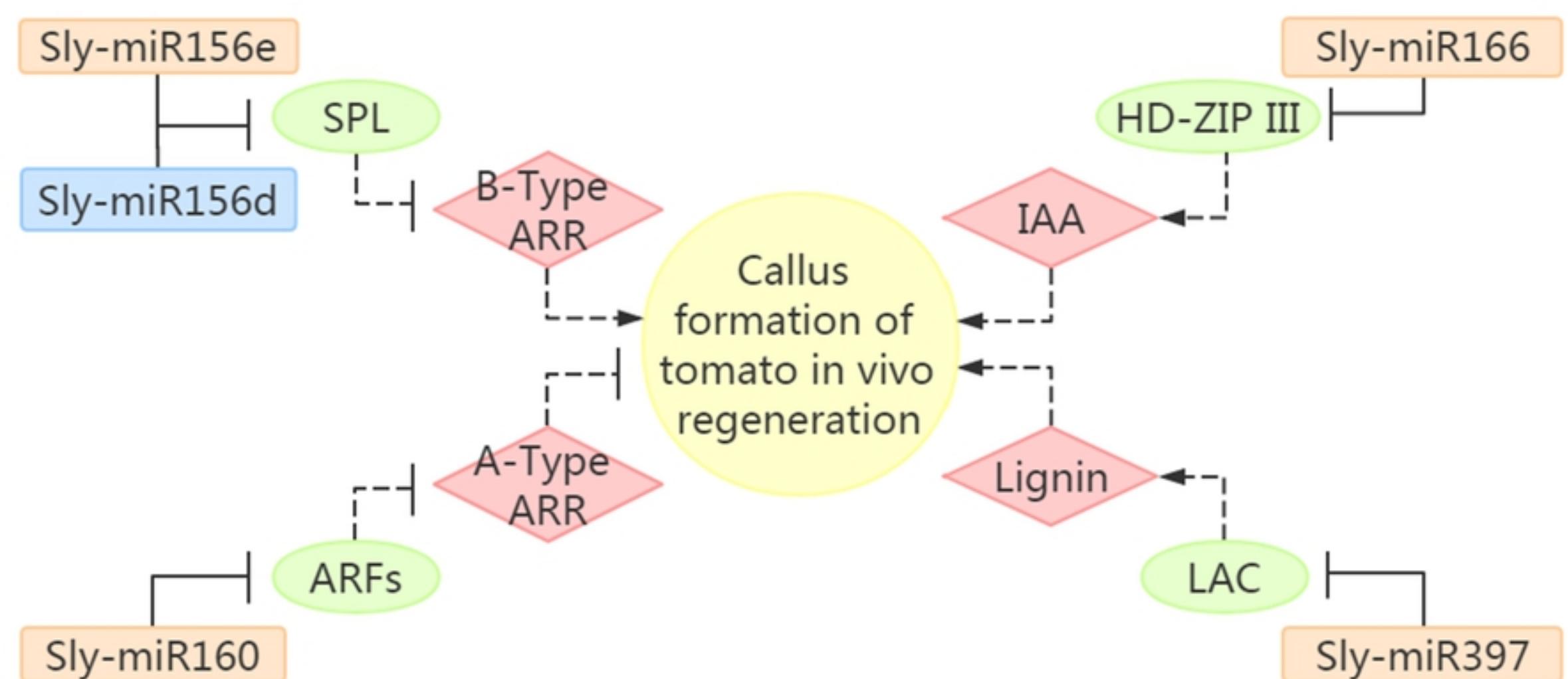
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(B)







Figure

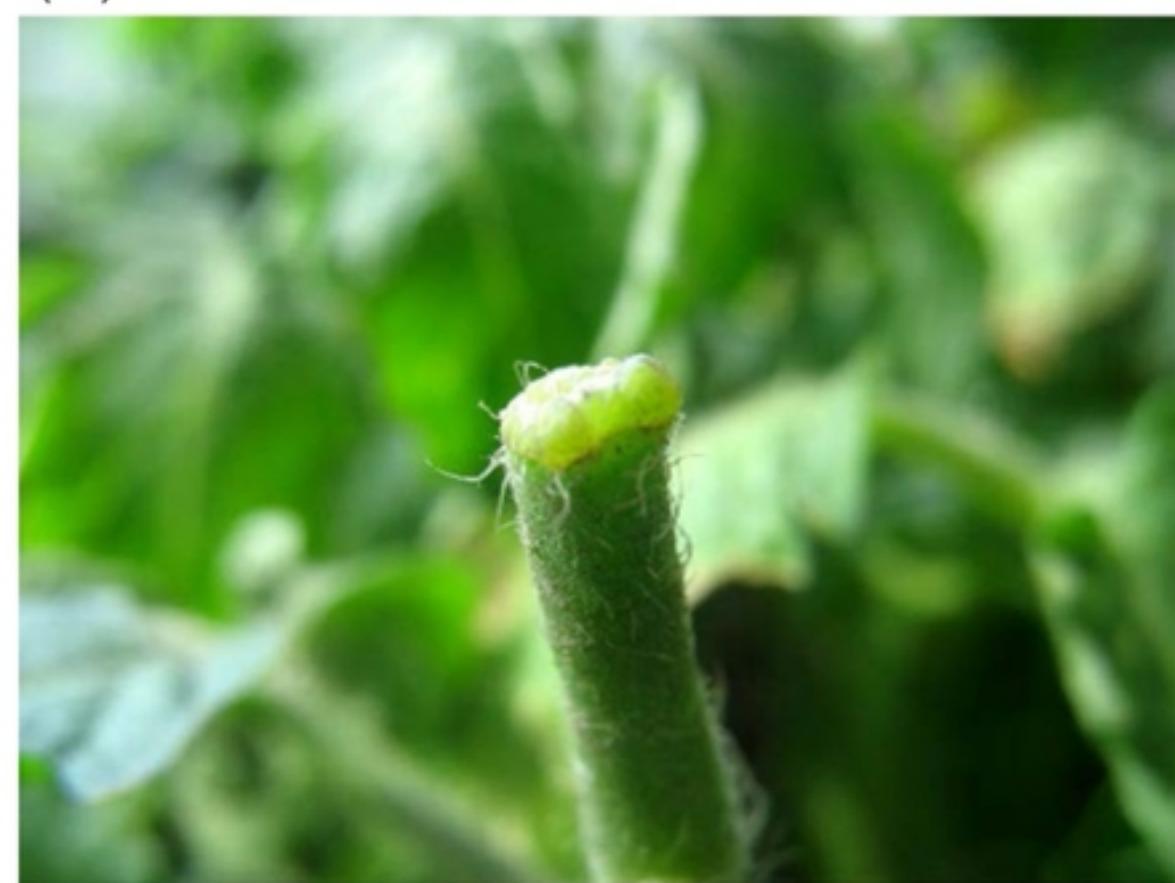
(A) (B)



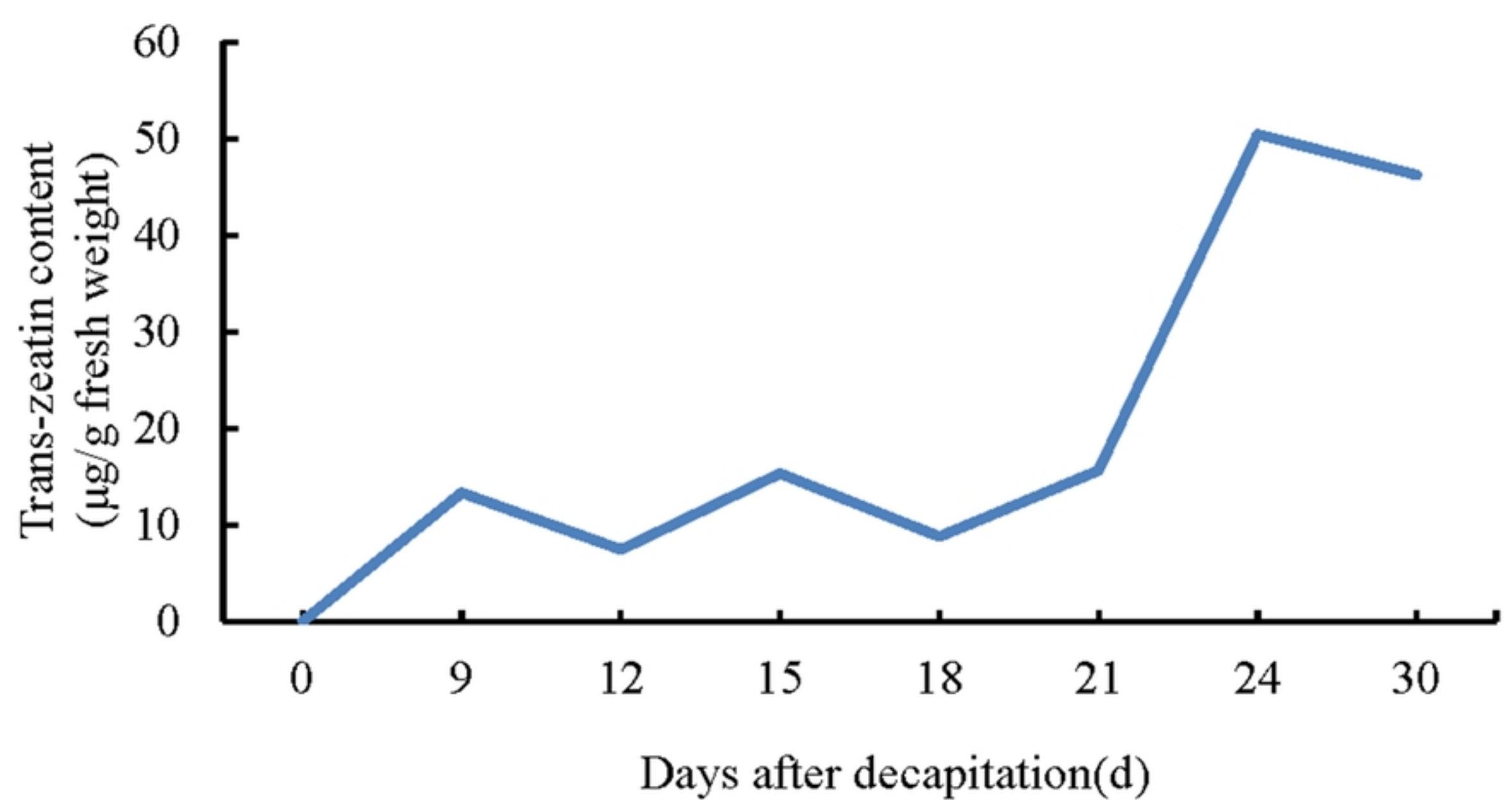
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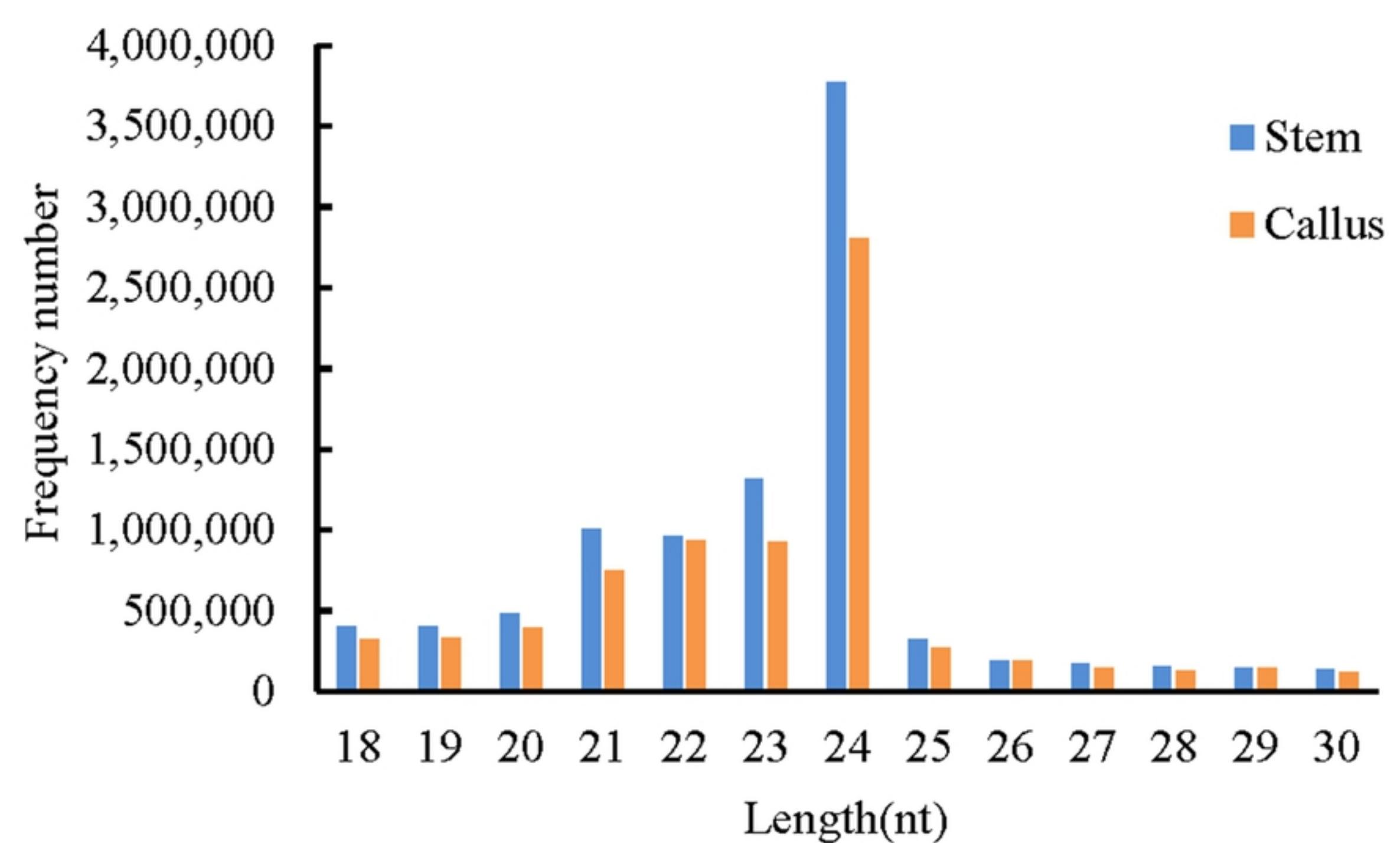
(C)



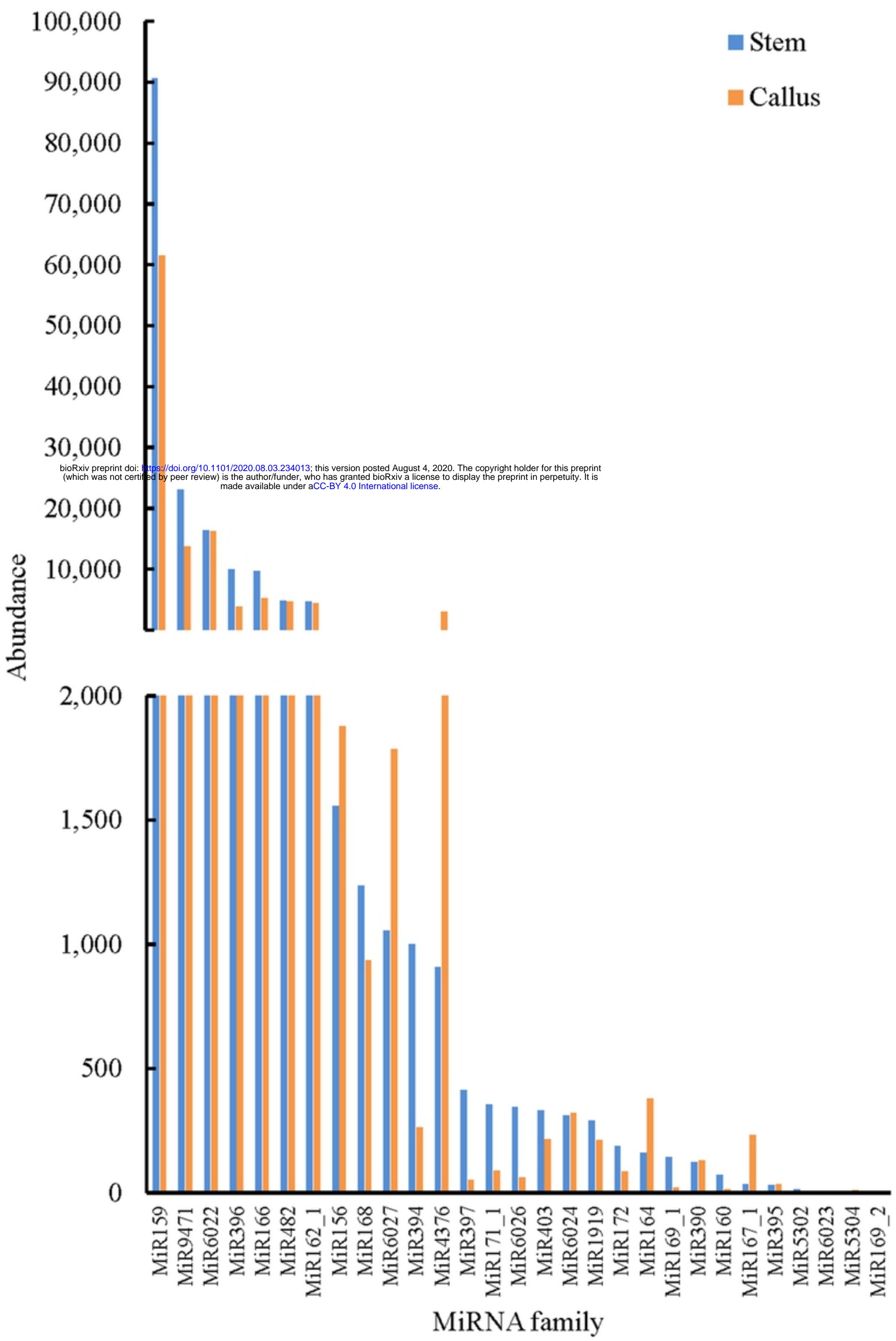
Figure



Figure



Figure



Figure

(A)

(B)

