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1 **Article title: Building a robust chromatin immunoprecipitation**
2 **(ChIP) method with substantially improved efficiency**
3
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20 **One sentence summary:**
21 Building a ChIP method that increases fold enrichment of birch by 16 folds in average and is
22 adapted for both woody and herbaceous plants.
23
24 **Authors' contributions**
25 YCW conceived and directed the project. HMZ and HYL performed the experiments;
26 HMZ, XJW, YQJ performed the overall data analysis; HMZ, HYX provides
27 Arabidopsis AST1 overexpression transgenic seeds; HYG provide MYB

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28 over-expressing strains; YCW wrote the manuscript; all authors revised the
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36
37

38 **ABSTRACT**

39 Chromatin immunoprecipitation (ChIP) is the gold-standard method to detect the
40 interactions between proteins and chromatin, and is a powerful tool to identify
41 epigenetic modifications. Although ChIP protocols for plant species have been
42 developed, many specific features of plants, especially woody plants, still hinder the
43 efficiency of immunoprecipitation, resulting inefficient ChIP enrichment. There is an
44 active demand for a highly efficient ChIP protocol. In the present study, we employed
45 *Betula platyphylla* (birch) and *Arabidopsis thaliana* as the research materials, and five
46 factors closely associated with ChIP efficiency were identified, including crosslinking,
47 chromatin concentration using centrifugal filter, using new immunoprecipitation
48 buffer, rescue DNA with proteinase K, and using sucrose to increase
49 immunoprecipitation efficiency. Optimization of any these factors can significantly
50 improve ChIP efficiency. Considering these factors together, a robust ChIP protocol
51 was developed, for which the average fold enrichments were 16.88 and 6.43 fold of
52 that gained using standard ChIP in birch and Arabidopsis, respectively. As this built
53 ChIP method works well in both birch and Arabidopsis, it should be also suitable for
54 other woody and herbaceous species. In addition, this ChIP method make it is possible
55 to detect low-abundance TF-DNA interactions, and may extend the application of
56 ChIP in plant kingdom.
57

58 **Key words:** *Arabidopsis thaliana*, *Betula platyphylla,* centrifugal filter, chromatin
59 immunoprecipitation, ChIP, cross-linking reversal, sucrose,

60

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61 **INTRODUCTION**

62 Chromatin immunoprecipitation (ChIP) is an important technique that is widely used
63 to examine epigenetic modifications or identify protein-DNA interactions.
64 Transcription factors (TFs) bind to regulatory sequences to modulate gene expression,
65 and gene expression regulation plays an essential role in various of cellular processes.
66 However, which genes are directly regulated by the TFs, and how the TFs control
67 gene expression *in vivo*, remain largely unknown. ChIP is a powerful tool to identify
68 genes that are regulated directly by certain TFs and to address TFs' recognition of
69 their target genes *in vivo*. In addition, combined with high throughput sequencing
70 technology, ChIP-seq has become the gold-standard method to detect binding regions
71 for TFs on a genome-wide scale (Verkest *et al.*, 2014). However, current ChIP
72 procedures have shortcomings in terms of the overall inefficiency of ChIP enrichment,
73 making it difficult to detect low-abundance TF-DNA interactions (Verkest *et al.*,
74 2014).

75 Although ChIP protocols for plant species have been developed (Bowler *et al.,*
76 2004, Gendrel *et al.,* 2005, Saleh *et al.,* 2008, Kaufmann *et al.,* 2010), some specific
77 features of plants species, such as rigid cell walls, chloroplasts, the paucity of nuclei
78 in some tissues, and large vacuoles, all markedly hinder the immunoprecipitation of
79 DNA and represent a challenge for TF-DNA enrichment. Therefore, genome-wide
80 ChIP studies of plant species are lagging behind those of other eukaryotic systems
81 (Verkest *et al.*, 2014). In addition, woody plants have thick-walled cells, and high
82 levels of phenolics and/or polysaccharides, which adversely affect many key steps in
83 ChIP procedures that have been optimized for tissues or cells of non-woody plants (Li
84 *et al.,* 2014). An alternative approach to ChIP, Chromatin Affinity Purification (ChAP),
85 had been proposed, which does not require immunoprecipitation, and is effective in
86 plant chromatin studies (Zentner et al., 2014). Additionally, tandem chromatin affinity
87 purification (TChAP) has also been developed in *Arabidopsis thaliana* plants, which
88 can greatly improve DNA enrichment efficiency compared with ChIP (Verkest *et al.*,
89 2014). However, both ChAP and TChAP cannot be used in epigenetic studies, such as
90 post-translational histone modifications, and standard ChIP is the most used method in

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91 epigenetics. At the same time, in the study of TF-DNA interactions, standard ChIP is
92 still widely used in most case studies because of its properties. Therefore, standard
93 ChIP is an important tool in molecular biology investigations. Standard ChIP does not
94 work well in some plant species, especially woody plants; therefore, there is an active
95 demand to overcome the inefficiency of ChIP enrichment, and to improve standard
96 ChIP to make it suitable for both woody and herbaceous plants.

97 In the present study, we studied the factors that influence the efficiency of standard
98 ChIP, and some key processes in the ChIP protocol were identified and optimized.
99 Consideration of the factors involved in ChIP efficiency allowed us to develop a ChIP
100 method that could significantly improve ChIP efficiency and could work well in
101 woody and herbaceous plants. In addition, the results obtained in the present study
102 could be used to develop an efficient ChIP protocol for use in other eukaryotic species,
103 and the strategies and technologies used to optimize ChIP could also be used in other
104 techniques that involve immunoprecipitation.

105

106

107 **RESULTS**

108 **Crosslinking using 3% formaldehyde can resist decrosslinking caused by**
109 **sonication better than 1% formaldehyde**

110 Decrosslinking of chromatin during ChIP will reduce the yield. Sonication
111 treatment can cause decrosslinking of chromatin; therefore, we first studied whether
112 different concentrations of formaldehyde treatment could affect the decrosslinking
113 caused by sonication. Birch plants (*Betula platyphylla*) were crosslinked using 1%
114 and 3% formaldehyde, respectively, and then treated with sonication for chromatin
115 fragment. After sonication, the decrosslinked DNA was harvested by extraction with
116 Tris-phenol and chloroform, and analyzed using quantitative real-time polymerase
117 chain reaction (qPCR). The results showed that 3% formaldehyde treatment could
118 reduce the decrosslinking of chromatin by 0.7–2.98 fold compared with 1%
119 formaldehyde treatment after sonication (Fig. 1). Therefore, considering the effects of

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120 decrosslinking chromatin caused by sonication, it is better to crosslink chromatin and
121 proteins using 3% formaldehyde rather than 1% formaldehyde.
122

123 The concentration of the crosslinked chromatin significantly increases the
124 immunoprecipitation efficiency

125 To study the effects of the concentration of formaldehyde-crosslinked-chromatin on
126 immunoprecipitation, the sonicated chromatin from transgenic birch overexpressing
127 *BplMYB46* was purified and concentrated using a 30 kDa cutoff centrifugal filter, and
128 then the same quantity of antibody for immunoprecipitation as standard ChIP method
129 was used. The standard ChIP procedure (described in the section of “Procedure for
130 standard ChIP”) was also performed as the control. ChIP-qPCR was performed to
131 check the efficiency of the enrichment of ChIP products. Four genes whose promoters
132 had been confirmed to be bound by BplMYB46 in birch were analyzed. After the
133 chromatin was concentrated using protein centrifugal filters, the enrichment increased
134 markedly to 2.01 to 3.31-fold higher than that gained without using the centrifugal
135 filter (Fig. 2). These results suggested that concentration of the crosslinked chromatin
136 using a protein centrifugal filter is quite important for immunoprecipitation, resulting
137 in a significant increase in immunoprecipitation efficiency and improving the
138 enrichment of ChIP DNA.
139

140 A new buffer for immunoprecipitation in ChIP

141 The immunoprecipitation buffer is very important for the immunoprecipitation and
142 enrichment of ChIP DNA. To optimize the immunoprecipitation buffer, we developed
143 a new buffer, termed optimized immunoprecipitation (OIP) buffer, which has a similar
144 pH value (pH = 7.4) to that of the plant nucleus (Shen et al., 2013). ChIP was
145 performed using the OIP buffer, and the classic IP buffer (ChIP Ab incubation buffer)
146 was used as a control. The ChIP-qPCR results showed that using the OIP buffer
147 improved the efficiency of ChIP significantly, with an enrichment of 2.58–4.72-fold
148 compared with that gained using the ChIP Ab incubation buffer (Fig. 3). These results
149 suggested that the OIP buffer is more efficient for immunoprecipitation than the ChIP

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150 Ab incubation buffer.

151

152 **Determination of the most suitable NaCl concentration for immunoprecipitation**

153 Previous research showed that 150 mM NaCl is not a suitable concentration for
154 immunoprecipitation (Li *et al.,* 2014); therefore, we determined whether 150 mM
155 NaCl is a suitable concentration for immunoprecipitation in the OIP buffer. OIP
156 buffers with NaCl at 100, 120, 150, and 170 mM were used, and the concentrations of
157 the other reagents in the OIP buffer are unchanged. The results showed that 150 mM
158 NaCl displayed highest immunoprecipitation efficiency, followed by 170 mM NaCl;
159 however, 100 and 120 mM NaCl showed relatively low immunoprecipitation
160 efficiency (Fig. 4). This result suggested that 150 mM NaCl is the most suitable
161 concentration for immunoprecipitation in the OIP buffer.

162

163 **Sucrose plays an important role in improvement of immunoprecipitation**

164 To determine whether sucrose is involved in immunoprecipitation efficiency, OIP
165 buffers containing 0, 5, 7, 9, and 11% (w/v) sucrose were made, and
166 immunoprecipitation was performed. ChIP-qPCR analysis was used to determine the
167 immunoprecipitation efficiency. The results showed that sucrose at 5–11% could
168 improve the efficiency of immunoprecipitation (Fig. 5). In addition, 7% sucrose had
169 the largest effect on immunoprecipitation efficiency, producing an enrichment of
170 3.54–7.41-fold compared with that of the control (Fig. 5). Therefore, sucrose plays an
171 important role in immunoprecipitation improvement, and 7% sucrose was identified
172 as the most suitable sucrose concentration for immunoprecipitation.

173

174 **Optimization of the crosslinking reversal step**

175 Next, we optimized the procedure for reversing the crosslinking between chromatin
176 and proteins. The ChIP procedure was performed according to the standard ChIP
177 protocol. After elution of the ChIP DNA, the elution product was divided into two
178 equal portions. Proteinase K was added to one portion for crosslinking reversal, and
179 the other portion was reverse crosslinked using NaCl overnight (standard protocol).

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180 The ChIP-qPCR results showed that the enrichment of ChIP DNA using Proteinase K
181 digestion increased by 2.41–3.20-fold compared with that achieved using the
182 NaCl-mediated classic crosslinking reversal procedure (Fig. 6).
183 We further compared three different crosslinking reversal methods: (1) proteinase K
184 direct treatment; (2) reverse crosslinking using NaCl; and (3) proteinase K treatment
185 after reversing crosslink using NaCl. Tris-phenol and chloroform extraction was
186 performed to remove the crosslinked DNA, and agarose gel electrophoresis was used
187 to monitor the amount of DNA released by crosslinking reversal. The results showed
188 that crosslinking reversal using NaCl overnight could not completely de-crosslink the
189 DNA; however, direct proteinase K digestion and proteinase K digestion after NaCl
190 crosslinking reversal could completely de-crosslink the DNA (Fig. 7). In addition,
191 compared with the other two approaches, direct proteinase K digestion was a time
192 saving and simpler method (Fig. 7).
193
194 **Building an improved ChIP protocol and determination of its**
195 **immunoprecipitation efficiency**
196 Based on the above results, we developed a new ChIP protocol. The procedures are
197 shown in Figure 8. The first improvement is that 3% formaldehyde was used for
198 chromatin crosslinking instead of 1% formaldehyde, which would reduce the
199 decrosslinking caused by sonication. The second improvement is that a protein
200 centrifugal filter was used to concentrate the chromatin. The third improvement was
201 the use of OIP buffer to substitute for classic ChIP Ab incubation buffer for
202 immunoprecipitation. The fourth improvement was the addition of sucrose to increase
203 immunoprecipitation. Finally, in the crosslinking reversal step, proteinase K was used
204 to directly digest proteins, which takes no more than 2 h and achieves complete
205 reversal of DNA crosslinking (Fig. 8).
206 Following this improved ChIP protocol, we determined its ChIP efficiency.
207 ChIP-qPCR showed that the fold enrichment using the improved ChIP method was
208 5.43–20.53-fold (average = 16.88-fold) higher than that achieved using standard ChIP
209 (Fig. 9). Consistently, repeating the experiment in Arabidopsis plants also showed an

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210 enrichment of 4.31–9.39-fold (average = 6.43-fold) using the improved ChIP method
211 compared with that achieved using the standard ChIP method (Fig. 9a, b). In addition,
212 the negative control genes were not enriched using the improved ChIP protocol
213 compared with that gained using the standard ChIP method, suggesting that the
214 improved ChIP method results in a lower background (Fig. 9a, b). At the same time,
215 when using the transgenic Arabidopsis plants expressing GFP gene only as material,
216 no fold enrichment of the aim DNA was observed (Fig. 9c), suggesting that this ChIP
217 method can specially enrich aim DNA. Taken together, these results indicated that the
218 improved ChIP procedure improved the efficiency of ChIP markedly.
219

220 **DISCUSSION**

221 We studied the factors that influence immunoprecipitation efficiency in ChIP, and
222 then built a robust ChIP protocol, which could significantly improve the enrichment
223 of ChIP DNA compared with that achieved using the standard ChIP protocol (Fig. 9).
224 Compared with the commonly used standard ChIP protocol and the protocol of Li
225 et al. (2014), this procedure has the following differences. (1) The use of 3%
226 formaldehyde for crosslinking to reduce de-crosslinking during sonication; (2)
227 concentration of crosslinked-chromatin using a centrifugal filter before
228 immunoprecipitation; (3) adding sucrose to increase the efficiency of the interaction
229 between the antibody and the antigen; (4) using a more suitable buffer for
230 immunoprecipitation; and (5) recovery of DNA from crosslinked chromatin using
231 proteinase K digestion instead of NaCl reversal.
232

233 **Concentration of crosslinked-chromatin is important for immunoprecipitation**

234 In the present study, protein ultrafiltration was employed to concentrate the
235 crosslinked-chromatin, which has the following four advantages: (1) This procedure
236 could eliminate some components released from cells that might inhibit the interaction
237 between the antigen and the antibody, and could also completely remove SDS that
238 will inhibit immunoprecipitation; (2) protein ultrafiltration can change the lysis buffer

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239 for the buffer that is most suitable for the antigen–antibody interaction, which would
240 greatly increase the efficiency of immunoprecipitation (Fig. 2); (3) the small
241 molecular weight (<100 kDa) proteins that do not cross-link with chromatin are
242 eliminated using ultrafiltration, which could reduce the background of
243 immunoprecipitation; and (4) this process can adjust the concentration of
244 crosslinked-chromatin to a suitable level for immunoprecipitation. By contrast, it is
245 difficult to solve the above four problems in the standard ChIP protocol. The results
246 showed that concentration of crosslinked chromatin using protein centrifugal filters
247 could significantly increase the efficiency of immunoprecipitation (Fig. 2). In addition,
248 in the present study, the concentration of crosslinked chromatin might not have been
249 the most appropriate for immunoprecipitation, and further adjustments to this
250 concentration might increase immunoprecipitation efficiency.
251
252 **Proteinase K digestion is efficient in rescue DNA from crosslinked chromatin**
253 Incubation of crosslinked chromatin in 0.2 M NaCl solution at 65 °C overnight is a
254 commonly used method to reverse DNA crosslinking. Some ChIP procedures also use
255 proteinase K for digestion after incubation in 0.2 M NaCl solution at 65 °C overnight
256 or for at least 6 h (Li *et al.,* 2014, Tsugama *et al.,* 2013, Haring *et al.,* 2007). In the
257 modified procedure, we used proteinase K to directly digest proteins and recover
258 DNA, which could be completed within 2 h. ChIP-qPCR showed that this method
259 increased the enrichment of ChIP products compared with using NaCl to reverse
260 crosslinking (Fig. 6), and has similar efficiency in DNA recovery compared with
261 proteinase K treatment after NaCl incubation (Fig. 6). However, direct proteinase K
262 treatment could save processing time. This method is simple and time saving;
263 therefore, it could be used commonly to reverse crosslinking in many experiments.
264
265 **OIP buffer can significantly improve immunoprecipitation efficiency**
266 The interactions between the antigen and the antibody is the key procedure in ChIP;
267 therefore, its optimization is important. In many ChIP protocols, the pH value of the
268 immunoprecipitation buffer is 8.0, which is higher than the pH of the nucleus;

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269 therefore, it might not be most appropriate for the immune interaction. In this study,
270 we employed HEPES-NaOH in the OIP buffer to achieve a pH value of 7.5, which is
271 close to the pH value in the nucleus according to Shen et al (2013). Moreover, some
272 reagents in the buffer were also optimized. Experiments showed that the OIP buffer
273 could improve the immunoprecipitation efficiency greatly compared with using the
274 ChIP Ab incubation buffer from the in standard ChIP protocol (Fig. 3). Therefore, the
275 OIP buffer is more suitable for use in ChIP in plants than the ChIP Ab incubation
276 buffer.
277

278 **Sucrose is important in improvement of immunoprecipitation**

279 To improve the efficiency of immunoprecipitation, we further studied other
280 reagents, including sucrose, trehalose, PEG, and glycerol (data not shown). Among
281 them, only sucrose displayed significantly increased efficiency of
282 immunoprecipitation. In addition, the results showed that 5–11% sucrose could
283 improve immunoprecipitation (Fig. 5). The role of sucrose in improving
284 immunoprecipitation has not been reported previously. Thus, to increase
285 immunoprecipitation efficiency sucrose could be used in experiments involving
286 interactions between the antibody and the antigen.
287

288 **The reason for using birch plants as researching material**

289 In the present study, we use birch as the plant material for ChIP investigation. Birch
290 is a woody plant species that is distributed widely in the cold temperate zone from
291 Europe to Asia, and shares many characteristics with other woody plants in terms of
292 its structure and composition. In addition, birch contains substantially higher levels of
293 chemical components compared with other woody plants, including polysaccharides
294 and polyphenols, which make it difficult to isolate DNA, RNA, or protein from this
295 plant (especially from the mature leaves of birch) compared with many other woody
296 plant species. Our developed ChIP protocol works well in birch; therefore, it should
297 also work well in other woody plant species. The improved ChIP works well in birch
298 and Arabidopsis; therefore, it could be adapted for use in other woody and herbaceous

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299 plants.

300 We should note that ChIP efficiency was improved more in birch than in
301 Arabidopsis using the improved ChIP protocol (Fig. 9). This might be explained by
302 the fact that birch has many kinds of metabolites, such as polysaccharides and
303 polyphenols, which will reduce immunoprecipitation efficiency quite highly than
304 Arabidopsis plants, leading to the standard ChIP procedure not working well.
305 Therefore, there is a large scope for improvement using the newly developed ChIP
306 protocol. However, in Arabidopsis, there are few metabolites that hinder the
307 immunoprecipitation efficiency compared with that in birch, and the standard ChIP
308 could work well, resulting limited improvement in ChIP efficiency compared with
309 that achieved in birch. Therefore, this improved ChIP protocol might be more useful
310 in the plants that have abundant metabolites that hinder immunoprecipitation
311 efficiency.

312

313 **CONCLUSIONS**

314 In the present study, an improved ChIP protocol was developed that includes five
315 improved procedures: (1) Crosslinking proteins and chromatin using 3%
316 formaldehyde instead of 1% formaldehyde; (2) concentration of crosslinked
317 chromatin; (3) using an optimized IP buffer; (4) the addition of sucrose to improve
318 immunoprecipitation efficiency; and (5) the use of proteinase K to digest proteins
319 crosslinked with DNA (Fig. 8). Improvement of each of the above procedures could
320 increase the enrichment of ChIP DNA significantly. Together, these five
321 improvements could increase the enrichment of ChIP DNA greatly. In addition, this
322 study might also provide helpful guidance to improve other experiments involving
323 immunoprecipitation.

324

325 **MATERIALS AND METHODS**

326 **Plant materials and the DNA sequences used in ChIP**

327 Two kinds of plant species were used as research materials, i.e. a woody plant
328 species, birch (*B. platyphylla*), and a herbaceous plant species, *A. thaliana*. Four genes

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329 that had been identified to be directly regulated by BplMYB46 in birch (Guo *et al.,*
330 2017), and birch plants overexpressing BplMYB46-FLAG (*BplMYB46*, Genbank
331 number: KP711284) were used in the ChIP experiments. Four genes that were directly
332 regulated by AST1 (*AST1*, AT3G24860) in *A. thaliana*(Xu *et al.,* 2018), and the
333 Arabidopsis plants overexpressing AST1-GFP were also used.
334

335 **Procedure for standard ChIP**

336 The ChIP procedure followed that of Haring et al (2007) with some modifications.
337 The detailed procedures were as follows: ***Plant material and crosslinking*****:** (1) One
338 gram of plant sample (aerial part; for birch sample used that is 5cm in height; for
339 Arabidopsis sample, 4-week-old T3 transgenic plants were used) was incubated in 30
340 ml of buffer A to cross-link the protein and DNA under vacuum conditions for 10 min.
341 Then, the sample was added with 2.5 ml of 2 M glycine, mixed well, and incubated
342 for 5 min to stop the cross-linking reaction. The solution was removed from the
343 sample, which was washed twice with cold milliQ water, and excess moisture was
344 thoroughly removed using paper towels. ***Nuclei isolation*****:** (2) The samples were
345 ground to a fine powder under liquid nitrogen, add with 30 ml of buffer B, and mixed
346 well. The following procedures were all performed on ice. (3) The solution was
347 filtered through four layers of Miracloth, and the filtrate was collected into a new 50
348 ml tube, and centrifuged for 20 min at 2,800 × *g* at 4 °C. The precipitates were
349 resuspended in 20 ml of buffer C, and the solution was centrifuged for 10 min at
350 12,000 × *g* and 4 °C; this step was repeated until the precipitate became white. (4) The
351 precipitate was resuspended in 300 μl of buffer D as the sample solution. Then, 600 μl
352 of buffer E was added to a new 1.5 ml tube, and overlaid with the sample solution
353 before centrifugation at 12000 × *g* for 45 min at 4 °C. ***Chromatin sonication*****:** (5) The
354 supernatant was removed and the pellet was resuspend in 320 μl of lysis buffer; a 10
355 μl aliquot sampled as “unsheared chromatin”. (6) The chromatin solution was sheared
356 into 200- to 1000-bp fragments using an ultrasonic homogenizer (Scientz-IID, Scientz
357 Biotechnology, Ningbo, China) with the following parameters: Sonication for 3 sec
358 and stopping for 15 sec, at 10% power setting for a total of 20 min. (7) The sonicated

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359 chromatin was centrifuged at 12,000 × *g* for 5 min at 4 °C. A 10 μl aliquot of the
360 chromatin solution was sampled to check the sonication efficiency. Then, a 50-μl
361 aliquot of the supernatant was reserved as the Input sample. ***Chromatin preclearing*****:**
362 (8) Then, 200 μl of the supernatant was diluted 10-fold by adding 1.8 ml of ChIP Ab
363 incubation buffer. Washed and blocked protein A/G Agarose beads (40 μl) were added
364 to the chromatin solution and the mixture was incubated for 1 h at 4 °C with gentle
365 agitation (12 rpm). The chromatin solution was then centrifuged at 1000 × g at 4 °C
366 for 5 min to pellet the beads. ***Preparation of washed and blocked protein A/G***
367 ***agarose beads*****:** (9) The supernatant of the protein A/G agarose beads (150 μl) was
368 removed by centrifugation at 1000 × *g* for 5 min, and 1 ml of ChIP Ab incubation
369 buffer was added. The beads were centrifuged at 1000 × *g* for 5 min to precipitate the
370 beads. This rinsing process was repeated twice. The beads were resuspended in 150 μl
371 of ChIP Ab incubation buffer with bovine serum albumin (BSA) to a final
372 concentration of 10 μg/ml. ***Immunoprecipitation*****:** (10) Two portions of 600 μl of the
373 chromatin solution from step 8 were taken, one portion was added with 6 μl of
374 anti-GFP or anti-FLAG antibodies as the ChIP sample (ChIP+), the other was added
375 with 6 μl anti-HA antibody as the mock control (ChIP-), and both were incubated at
376 4 °C overnight. (10) Washed protein A/G Agarose beads (60 μl) were added to the two
377 tubes, respectively. The tubes were incubated for 3 h at 4 °C with gentle agitation. (12)
378 The tubes were centrifuged for 5 minutes at 1000 × g at 4 °C to precipitate the beads.
379 ***Washing*****:** (13) The beads were washed for 10 min sequentially with 1 ml of the
380 following buffers: Low Salt Buffer, high salt buffer, LiCl wash buffer, and TE buffer.
381 (14) Then, 250 μl of prewarmed (65 °C) elution buffer (50mM Tris pH 8.0, 10 mM
382 EDTA, 1% SDS) was added, and the beads were resuspended by tapping and
383 incubated at room temperature for 15 min (mixing at 5-min intervals). The beads were
384 centrifuged for 5 minutes at 2000 × *g*, the supernatant was transferred into a new tube,
385 the elution step was repeated once, and the two eluates were mixed together. ***Reverse***
386 ***crosslinking*****:** (15) 5 M NaCl solution was added to the IP or mock control elution to
387 achieve a concentration of 0.2 M NaCl. Then, 100 μl of TE buffer was added to the
388 input sample, and 5 M NaCl was added to a concentration of 0.2 M NaCl. All samples

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389 were reverse cross-linked overnight at 65 °C. ***DNA purification*:** (16) The reverse
390 crosslinked samples were purified using a DNA purification spin column (Qiagen,
391 Hilden, Germany), and the column was eluted twice with 60 μl of TE buffer. Fold
392 enrichment calculation was following Haring et al (2007).
393

394 **Analysis of chromatin decrosslinking caused by sonication**

395 To investigate the effects of different concentrations of formaldehyde on chromatin
396 decrosslinking caused by sonication, birch plant samples were crosslinked with 1% or
397 3% formaldehyde and treated by sonication as described in "Procedure for standard
398 ChIP". After sonication, the decrosslinked chromatin was harvested by extraction with
399 an equal volume of Tris-phenol and chloroform, and the supernatant was further
400 extracted using an equal volume of chloroform. Then, the decrosslinked DNA was
401 harvested from the supernatant using a PCR purification kit (Qiagen). The extent of
402 DNA decrosslinking was analyzed using quantitative PCR.
403

404 **Analysis of the effects of concentration of crosslinked chromatin**

405 To concentrate the crosslinked chromatin, the chromatin was sonicated, centrifuged
406 (according to step 7 in the above ChIP protocol), and diluted 10-fold by adding ChIP
407 Ab incubation buffer (which dilutes the SDS to 0.1% to avoid micelle formation,
408 which would affect protein ultrafiltration), and the sonicated chromatin was purified
409 using protein centrifugal filters (30 kDa cutoff, Millipore, Billerica, MA, USA) to 300
410 μl, then added with 2700 μl of ChIP Ab incubation buffer, mixed well, and
411 concentrated with centrifugal filters to 300 μl again (following its manufacturer
412 instructions). The purified chromatin was divided into two equal portions. One was
413 used for immunoprecipitation (ChIP+) and the other was used as a no antibody
414 immunoprecipitation (ChIP-) control. The same volume of antibody as was used in
415 the classic protocol was added for immunoprecipitation. The subsequent steps were
416 same as those in the standard ChIP procedure. ChIP-qPCR was performed to study the
417 effects of protein centrifugal filter concentration.
418

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419 **Analysis of the ChIP efficiency using an optimized immunoprecipitation buffer**

420 The OIP buffer (20 mM HEEPS-NaOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1%
421 TritonX-100, 1 mM PMSF, proteinase inhibitors at 1 μg/ml each) was used to replace
422 the ChIP Ab incubation buffer in the immunoprecipitation step. Other procedures and
423 buffers were the same as those in the classic procedure described in above.
424 ChIP-qPCR was used to compare the immunoprecipitation efficiency between using
425 OIP and ChIP Ab incubation buffers.

426

427 **Analysis of the effects of different concentrations of NaCl in OIP buffer**

428 To determine the optimal concentration of NaCl for immunoprecipitation, NaCl in
429 the OIP buffer was used at 110, 120, 150, and 170 mM, and other reagents in OIP
430 buffer were unchanged. Other procedures and buffers were the same as the classic
431 procedure described in above. ChIP-qPCR was performed to investigate the most
432 suitable NaCl concentration for immunoprecipitation.

433

434 **Investigation of the effect of sucrose on immunoprecipitation**

435 To determine the effect of sucrose on immunoprecipitation, sucrose at 5, 7, 9 and
436 11% (w/v) were added into the OIP buffer for immunoprecipitation. The other steps of
437 ChIP followed the standard ChIP protocol. ChIP-qPCR was performed to study
438 whether sucrose at different concentrations affected ChIP immunoprecipitation.

439

440 **Comparison of different methods of crosslink reversal**

441 Two methods to recover DNA from crosslinked chromatin were compared. After
442 the ChIP product elution (step 13), the eluted product was divided into two equal
443 portions. Portion one was added with Proteinase K to a final concentration of 0.7
444 μg/μl and incubated at 55 °C for 2 h to decompose the crosslink between the protein
445 and chromatin. Portion two was subjected to chromatin crosslinking reversal
446 following the classic procedure, i.e., added with NaCl to a final concentration of 0.2
447 M, and incubated at 65 °C overnight.

448 Then, DNA was purified from these two portions using a DNA purification spin

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449 column (Qiagen), and eluted with 60 μl of TE buffer. To compare these two methods
450 of recovery of DNA from crosslinked chromatin, qPCR was performed.
451

452 **Comparison of the methods to recover DNA from crosslinked chromatin**

453 Three methods to purify DNA from crosslinked chromatin were compared. After
454 elution of the ChIP products (step 13), the eluted products were divided into three
455 equal portions. Method one used reverse crosslinking from the classic method, i.e.
456 following the procedure described at step 14 (as a control). In method two, the sample
457 was added with proteinase K to a final concentration of 0.7 μg/μl and incubated at
458 55 °C for 2 h. In method three, crosslinking reversal was performed according to the
459 method of Li et al (2014), i.e. NaCl to the final concentration of 0.2 M was added, and
460 the sample was incubated at 65 °C for 6 h to reverse cross-linking. Then, 32.5 μl of
461 protease/RNase buffer (150 mM EDTA (pH 8.0), 615 mM Tris-HCl (pH 6.5), 14
462 mg/ml proteinase K, and 0.30 μg/μl RNase) was added and the mixture was incubated
463 at 45 °C for 1 h.

464 The products from these three methods were extracted separately with an equal
465 volume of Tris-phenol (pH 8.0) and chloroform (1:1 v/v), and then extracted with an
466 equal volume of chloroform. Ten microliters of supernatant was electrophoresed
467 through an agarose gel to determine the quantity of DNA.
468

469 **The protocol of the newly developed ChIP technique**

470 The improved ChIP protocol was developed (Fig. 6) using the following procedures.
471 Step 1 used 3% formaldehyde instead of 1% formaldehyde for chromatin crosslinking.
472 Step 2–7 were the same as classic protocol described above. In step 8, 200 μl of the
473 supernatant was diluted 10-fold by adding 1.8 ml of OIP buffer (supplemented with
474 7% sucrose), and was concentrated using protein centrifugal filters (30 kDa cutoff) to
475 300 μl. Then, a 2700 μl of OIP buffer (supplemented with 7% sucrose) was added and
476 mixed well. The sample was then concentrated using centrifugal filters to 400 μl. In
477 step 10, two portions of 200 μl of the chromatin solution from step 8 were taken. One
478 portion was added with 6 μl of the target antibody as the ChIP sample (ChIP+), the

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479 other portion had antibody added (or was added with another antibody) as the mock
480 control (ChIP-), and incubated at 4 °C overnight. Steps 11–14 followed the classic
481 protocol. In step 15, the elution buffer was added with proteinase K to a final
482 concentration of 0.2 μg/μl and incubated at 55 °C for 2 h. Step 16 was the same as the
483 classic protocol. The detailed protocol of developed ChIP was shown as
484 Supplementary file 1.
485
486 **Quantitative real-time PCR analysis**
487 Quantitative real-time PCR was performed on a qTower 2.2 (Analytik Jena AG, Jena,
488 Germany). The PCR reaction system contained 10 μl of SYBR Green Real-time PCR
489 Master Mix (Toyobo, Osaka, Japan), 0.5 μM of each forward and reverse primes, and
490 2 μL of ChIP product as the PCR template, with a total volume of 20 μl. The PCR
491 reaction was conducted with the following parameters: 94 °C for 1 min; 40 cycles of
492 94 °C for 12 s, 58 °C for 30 s, and 72 °C for 45 s. Melting curves were generated for
493 each reaction to identify the specificity of the PCR reaction. The DNA sequence of
494 ubiquitin (FG065618) and ACT7 (AT5G09810) were used as the internal controls in
495 birch and Arabidopsis, respectively. The primers for ChIP-PCR used in birch were the
496 same as those used by Guo et al (2017), and the primers used in Arabidopsis were the
497 same as those used by Xu et al (2018). The primers used for ChIP-qPCR are shown in
498 Table S1.
499
500 **Reagents**
501 Sucrose (Sigma-Aldrich); Formaldehyde solution 37% (wt/wt) (Sigma-Aldrich);
502 Glycine (Fisher Scientific); Tris base (Promega); Hydrochloric acid (HCl;
503 Sigma-Aldrich); PMSF, aprotinin, leupeptin, and pepstatin A (Sigma-Aldrich); DMSO
504 (Sigma-Aldrich); β-Mercaptoethanol (Sigma-Aldrich); Miracloth (Calbiochem, San
505 Diego, CA, USA); anti-FLAG antibody (Sigma-Aldrich , SAB4301135); anti-GFP
506 antibody(Sigma-Aldrich, SAB4301138); protein A/G Agarose beads (Beyotime
507 Biotechnology, Shanghai, China); Triton X-100 (Solarbio); EDTA disodium salt
508 (Solarbio); Proteinase K (Promega); RNase A solution (Promega).

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509

510 **ChIP Buffers**

511 Buffer A comprised 10 mM Tris-HCl (pH 8.0), 0.4 M sucrose, 1.0 % w/v
512 formaldehyde, 1 mM PMSF, proteinase inhibitors (aprotinin, leupeptin, pepstatin A , 1
513 μg/ml each), and 5 mM β-mercaptoethanol.
514 Buffer B comprised 10 mM Tris-HCl (pH 8.0), 0.4 M sucrose, 5 mM $MgCl_2$, 5 mM
515 β-mercaptoethanol, 1 mM PMSF, and proteinase inhibitors (aprotinin, leupeptin,
516 pepstatin A) at 1 μg/ml each.
517 Buffer C comprised 10 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 10 mM $MgCl_2$, 1.2%
518 Triton X-100, 5 mM 2-mercaptoethanol, 1 mM PMSF, and proteinase inhibitors
519 (aprotinin, leupeptin, pepstatin A) at 1 μg/ml each.
520 Buffer D comprised 10 mM Tris/HCl pH 8.0, 0.7 M sucrose, 2 mM $MgCl_2$, 5 mM
521 2-mercaptoethanol, 1 mM PMSF, and proteinase inhibitors (aprotinin, leupeptin,
522 pepstatin A) at 1 μg/ml each.
523 Buffer E comprised (10 mM Tris/HCl pH 8.0, 2.0 M sucrose, 2 mM $MgCl_2$, 5 mM
524 2-mercaptoethanol, 1 mM PMSF, and proteinase inhibitors (aprotinin, leupeptin,
525 pepstatin A) at 1 μg/ml each.
526 Lysis buffer comprised 50 mM Tris pH 8.0, 10 mM EDTA, 1 % w/v SDS, 1 mM
527 PMSF, and proteinase inhibitors (aprotinin, leupeptin, pepstatin A) at 1 μg/ml each.
528 ChIP Ab incubation buffer comprised 20 mM Tris pH 8.0, 1.2 mM EDTA, 1.2% v/v
529 Triton X-100, 150 mM NaCl, 1 mM PMSF, and proteinase inhibitors (aprotinin,
530 leupeptin, pepstatin A) 1 μg/ml each.
531 Low Salt Buffer comprised 20 mM Tris pH 8.0, 2 mM EDTA, 0.1% w/v SDS, 1% v/v
532 Triton X-100, and 150 mM NaCl).
533 High salt buffer comprised 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 1%
534 Triton X-100, and 1 mM EDTA) (wash three times);
535 LiCl wash buffer comprised 25 mM LiCl, 0.1% sodium deoxycholate, 1 mM EDTA,
536 and 20 mM Tris-HCl, pH 8.0 (two washes).
537 TE buffer comprised 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (two washes).
538 Elution buffer comprised 50 mM Tris pH 8.0, 10 mM EDTA, and 1% SDS.

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539

540 **ACKNOWLEDGMENTS**

541 Not applicable.

542

543 **CONSENT FOR PUBLICATION**

544 Not applicable.

545

546 **COMPETING INTERESTS**

547 The authors declare that they have no competing interests.

548

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552

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592

593 **Supplemental materials**

594 **Supplemental file 1**. The procedure of new developed ChIP.

595 **Supplemental Table 1.** The primers used in ChIP-qPCR

596

597 **Figure legends**

598 **Figure 1. Comparison of decrosslinking caused by sonication between chromatin**
599 **crosslinked by 1% and 3% formaldehyde.**

600 Four truncated promoters from birch that had been confirmed to be directly bound by
601 BplMYB46 were studied. The chromatin was crosslinked with 1% or 3%
602 formaldehyde, and treated with sonication. After sonication, the de-crosslinked DNA
603 was harvested by extraction with Tris-phenol and chloroform, and analyzed using
604 qPCR. Three independent experiments were performed, and data are means ± SD
605 from three replicates.

606

607 **Figure 2. Concentration of chromatin using protein centrifugal filters significantly**
608 **improves ChIP efficiency.**

609 Formaldehyde crosslinked chromatin was purified and concentrated using protein
610 ultrafiltration centrifugal tube, and then was used for immunoprecipitation. Con: Fold

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611 enrichment in the standard ChIP protocol. Concentration: Fold enrichment of ChIP
612 performed with concentrated chromatin with centrifugal filter. The relative fold
613 enrichment was calculated as: Concentration/Con. Four truncated promoters of genes
614 from *B. platyphylla* that were previously confirmed to be directly regulated by
615 *BplMYB46* were analyzed, and their fold enrichments were determined using
616 ChIP-qPCR. Three independent experiments were performed, and data are means ±
617 SD from three replicates.
618
619 **Figure 3. Determination of ChIP efficiency using the optimized**
620 **immunoprecipitation buffer.**
621 The OIP (optimized immunoprecipitation) buffer was used for immunoprecipitation,
622 and the immunoprecipitation buffer from the standard ChIP protocol was used as a
623 control. Con: Fold enrichment of ChIP performed using ChIP Ab incubation buffer
624 from the classic protocol, which was used as control; OIP: Fold enrichment of ChIP
625 performed using the OIP buffer. The relative fold enrichment was calculated as:
626 OIP/Con. Four truncated promoters of genes directly regulated by *BplMYB46* were
627 studied in *B. platyphylla* using ChIP-qPCR. Three independent experiments were
628 performed, and data are means ± SD from three replicates.
629
630 **Figure 4. Determination of the optimum NaCl concentration for**
631 **immunoprecipitation.**
632 Different concentrations of NaCl in the OIP (optimized immunoprecipitation) buffer
633 were studied for ChIP immunoprecipitation, and ChIP-qPCR was performed to
634 determine the fold enrichment Four truncated promoters of genes in *B. platyphylla*
635 were studied using ChIP-qPCR. Three independent experiments were performed, and
636 data are means ± SD from three replicates.
637
638 **Figure 5. Determination of the effects of sucrose on immunoprecipitation**
639 **efficiency.**

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640 Different concentrations of sucrose were added into the OIP (optimized
641 immunoprecipitation) buffer, and immunoprecipitation was performed. Con: Fold
642 enrichment of ChIP performed using OIP buffer without sucrose (control). 5, 7, 9 and
643 11% sucrose: fold enrichment of ChIP performed using 5, 7, 9 and 11% of sucrose in
644 the OIP buffer. The relative fold enrichment was calculated as 5, 7, 9 and 11%/Con.
645 Four truncated promoters of genes directly regulated by *BplMYB46* were studied in *B.*
646 *platyphylla* using ChIP-qPCR. Three independent experiments were performed, and
647 data are means ± SD from three replicates.
648
649 **Figure 6. Comparison of methods of crosslinking reversal in ChIP.**
650 ChIP was performed according to the classic protocol and the eluted ChIP DNA was
651 divided equally into two portions, which were used for crosslinking reversal using two
652 methods. Method 1: Proteinase K direct digestion to substitute for crosslinking
653 reversal; method 2: crosslinking reversal using NaCl at 65 °C overnight. ChIP-qPCR
654 was conducted to determine the amounts of ChIP DNA. Proteinase K: Fold
655 enrichment of ChIP performed using method 1; Con: Fold enrichment of ChIP
656 performed using method 2 as the control. The relative ChIP fold enrichment was
657 calculated as: Proteinase K/Con. Four truncated promoters of genes directly regulated
658 by *BplMYB46* were studied in *B. platyphylla* using ChIP-qPCR. Three independent
659 experiments were performed, and data are means ± SD from three replicates.
660
661 **Figure 7. Determination of the efficiency of different crosslinking reversal**
662 **methods.**
663 Three methods were performed on birch chromatin. Method 1: Reversal of
664 crosslinking using NaCl at 65 °C overnight; method 2: Proteinase K digestion after
665 crosslinking reversal using NaCl at 65 °C for 6 h; method 3: Proteinase K direct
666 digestion at 55 °C for 2 h. +DC: samples were decrosslinked. -DC: samples were not
667 decrosslinked. After these three methods were performed, the chromatin was extracted
668 using one volume of Tris-phenol and chloroform (1:1 v/v), followed by extraction
669 using one volume of chloroform. The supernatant was electrophoresed through an

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670 agarose gel to determine the quantity of decrosslinked DNA. M: DNA marker; Line 1,
671 2, 3: Crosslinking reversal of chromatin using methods 1, 2, and 3, respectively.
672
673 **Figure 8. The procedure for the newly developed ChIP protocol.**
674 The outline of the developed ChIP. a: The procedures for standard ChIP; b: The
675 procedures for the newly developed ChIP. The optimized procedures are marked with
676 red frames, which included crosslinking protein and chromatin using 3%
677 formaldehyde instead of 1% formaldehyde; purification and concentration of
678 chromatin using protein centrifugal filters; using OIP (optimized immunoprecipitation)
679 buffer instead of standard ChIP Ab incubation buffer for immunoprecipitation;
680 sucrose was added to improve the immunoprecipitation efficiency, and crosslinking
681 reversal was achieved using proteinase K directly. The detailed procedures are shown
682 as supplementary file 1.
683
684 **Figure 9. Analysis of the developed ChIP protocol for ChIP enrichment.**
685 ChIP was carried out using the improved ChIP protocol and the standard ChIP
686 protocol. The fold enrichment of ChIP was studied using ChIP-qPCR. Classic: fold
687 enrichment of ChIP performed using the standard ChIP protocol; New: fold
688 enrichment of ChIP performed using the new ChIP protocol. The relatively ChIP fold
689 enrichment were calculated as: New/Classic. (a, b) The ChIP fold enrichment values
690 were compared between the classic and the new ChIP protocol in *B. platyphylla* (a)
691 and *A. thaliana* (b). c: The Arabidopsis plants overexpression of GFP were used as
692 material for ChIP, and immunoprecipitation was performed with antiGFP antibidy (as
693 negative control). Four truncated promoters of genes from *B. platyphylla* and four
694 truncated promoters of genes from *A. thaliana* were analyzed using ChIP-qPCR.
695 Three independent experiments were performed, and data are means ± SD from three
696 replicates.
697

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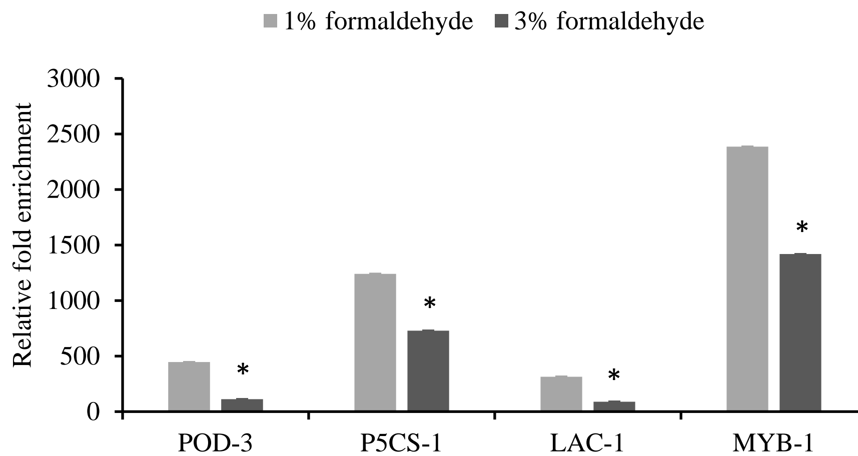

Figure 1. Comparison of decrosslinking caused by sonication between chromatin crosslinked by 1% and 3% formaldehyde.

Four truncated promoters from birch that had been confirmed to be directly bound by BplMYB46 were studied. The chromatin was crosslinked with 1% or 3% formaldehyde, and treated with sonication. After sonication, the de-crosslinked DNA was harvested by extraction with Tris-phenol and chloroform, and analyzed using qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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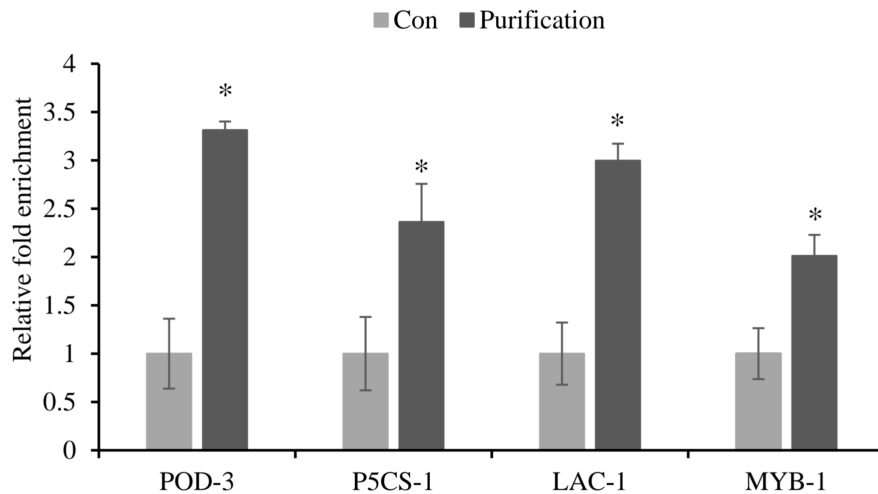

Figure 2. Concentration of chromatin using protein centrifugal filters significantly improves ChIP efficiency.

Formaldehyde crosslinked chromatin was purified and concentrated using protein ultrafiltration centrifugal tube, and then was used for immunoprecipitation. Con: Fold enrichment in the standard ChIP protocol. Concentration: Fold enrichment of ChIP performed with concentrated chromatin with centrifugal filter. The relative fold enrichment was calculated as: Concentration/Con. Four truncated promoters of genes from *B. platyphylla* that were previously confirmed to be directly regulated by *BplMYB46* were analyzed, and their fold enrichments were determined using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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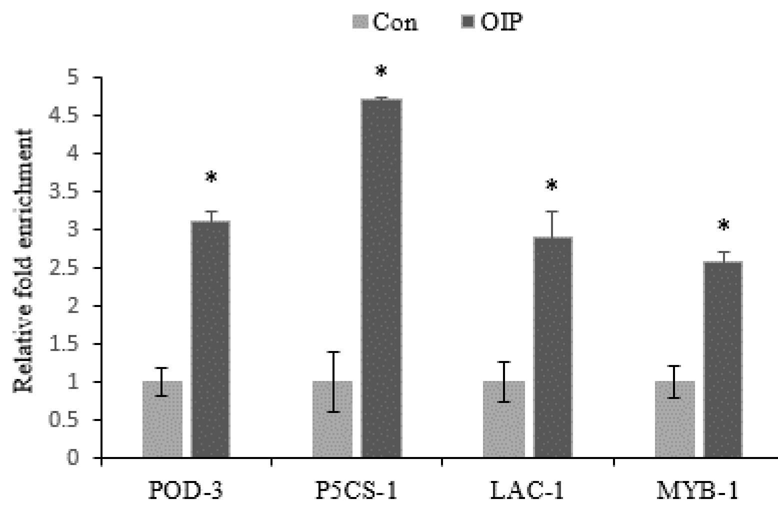

Figure 3. Determination of ChIP efficiency using the optimized immunoprecipitation buffer.

The OIP (optimized immunoprecipitation) buffer was used for immunoprecipitation, and the immunoprecipitation buffer from the standard ChIP protocol was used as a control. Con: Fold enrichment of ChIP performed using ChIP Ab incubation buffer from the classic protocol, which was used as control; OIP: Fold enrichment of ChIP performed using the OIP buffer. The relative fold enrichment was calculated as: OIP/Con. Four truncated promoters of genes directly regulated by *BplMYB46* were studied in *B. platyphylla* using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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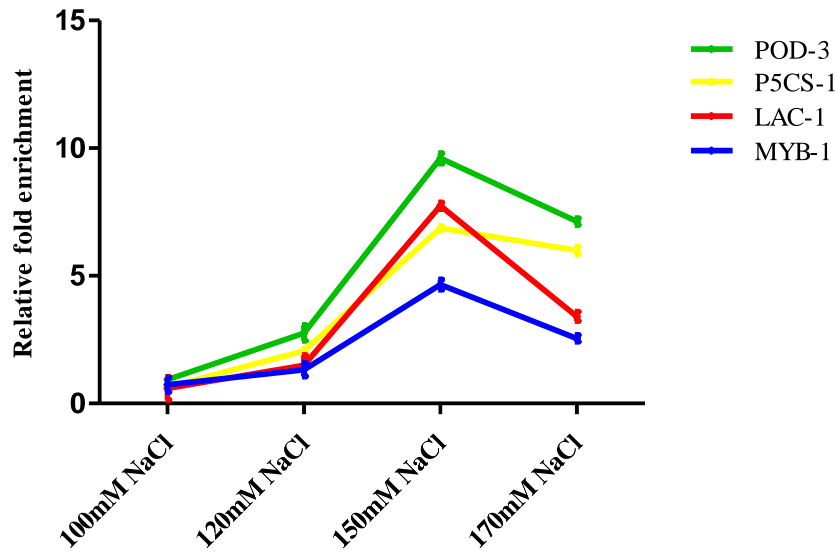

Figure 4. Determination of the optimum NaCl concentration for immunoprecipitation. Different concentrations of NaCl in the OIP (optimized immunoprecipitation) buffer were studied for ChIP immunoprecipitation, and ChIP-qPCR was performed to determine the fold enrichment Four truncated promoters of genes in *B. platyphylla* were studied using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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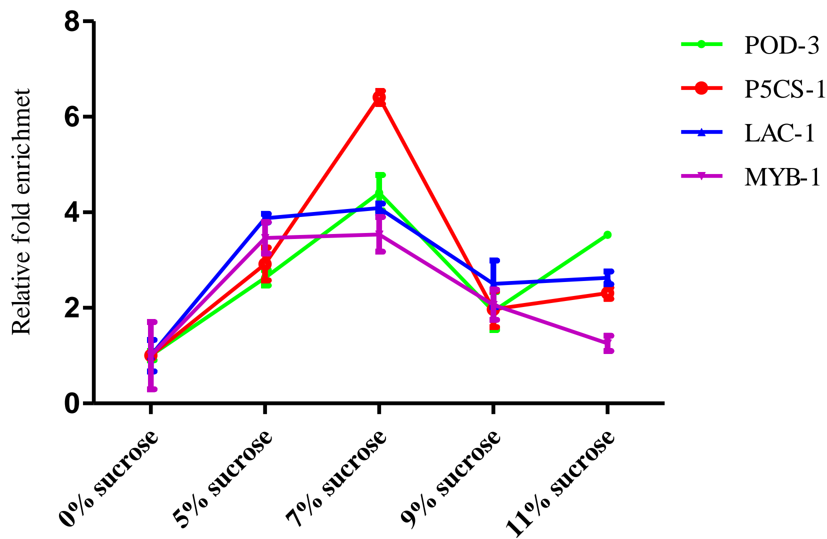

Figure 5. Determination of the effects of sucrose on immunoprecipitation efficiency. Different concentrations of sucrose were added into the OIP (optimized immunoprecipitation) buffer, and immunoprecipitation was performed. Con: Fold enrichment of ChIP performed using OIP buffer without sucrose (control). 5, 7, 9 and 11% sucrose: fold enrichment of ChIP performed using 5, 7, 9 and 11% of sucrose in the OIP buffer. The relative fold enrichment was calculated as 5, 7, 9 and 11%/Con. Four truncated promoters of genes directly regulated by *BplMYB46* were studied in *B. platyphylla* using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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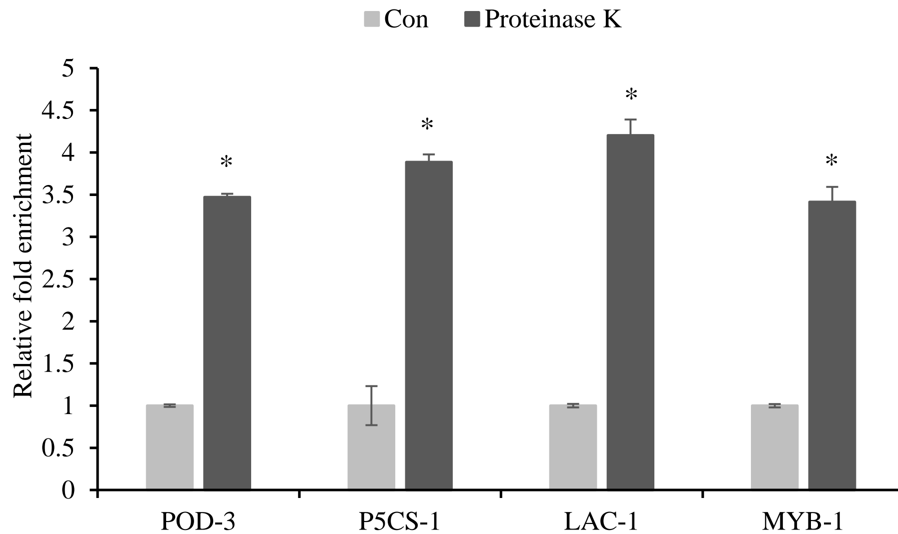

Figure 6. Comparison of methods of crosslinking reversal in ChIP.

ChIP was performed according to the classic protocol and the eluted ChIP DNA was divided equally into two portions, which were used for crosslinking reversal using two methods. Method 1: Proteinase K direct digestion to substitute for crosslinking reversal; method 2: crosslinking reversal using NaCl at 65 °C overnight. ChIP-qPCR was conducted to determine the amounts of ChIP DNA. Proteinase K: Fold enrichment of ChIP performed using method 1; Con: Fold enrichment of ChIP performed using method 2 as the control. The relative ChIP fold enrichment was calculated as: Proteinase K/Con. Four truncated promoters of genes directly regulated by *BplMYB46* were studied in *B. platyphylla* using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

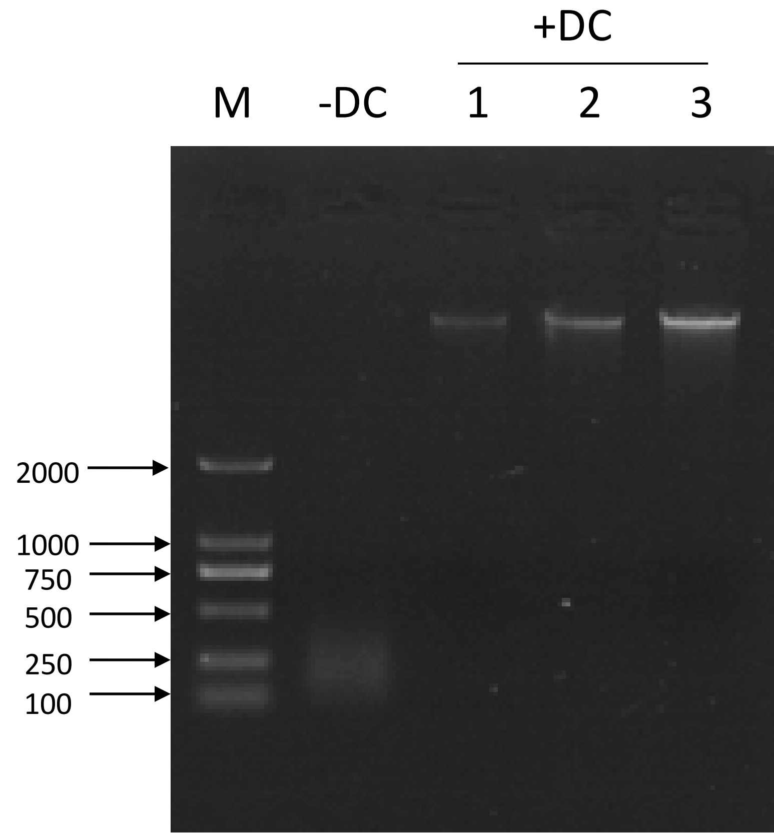

Figure 7. Determination of the efficiency of different crosslinking reversal methods.
Three methods were performed on birch chromatin. Method 1: Reversal of crosslinking using NaCl at 65 ° C overnight; method 2: Proteinase K digestion after crosslinking reversal using NaCl at 65 ° C for 6 h; method 3: Proteinase K direct digestion at 55 ° C for 2 h. +DC: samples were decrosslinked. -DC: samples were not decrosslinked. After these three methods were performed, the chromatin was extracted using one volume of Tris-phenol and chloroform (1:1 v/v), followed by extraction using one volume of chloroform. The supernatant was electrophoresed through an agarose gel to determine the quantity of decrosslinked DNA. M: DNA marker; Line 1, 2, 3: Crosslinking reversal of chromatin using methods 1, 2, and 3, respectively.

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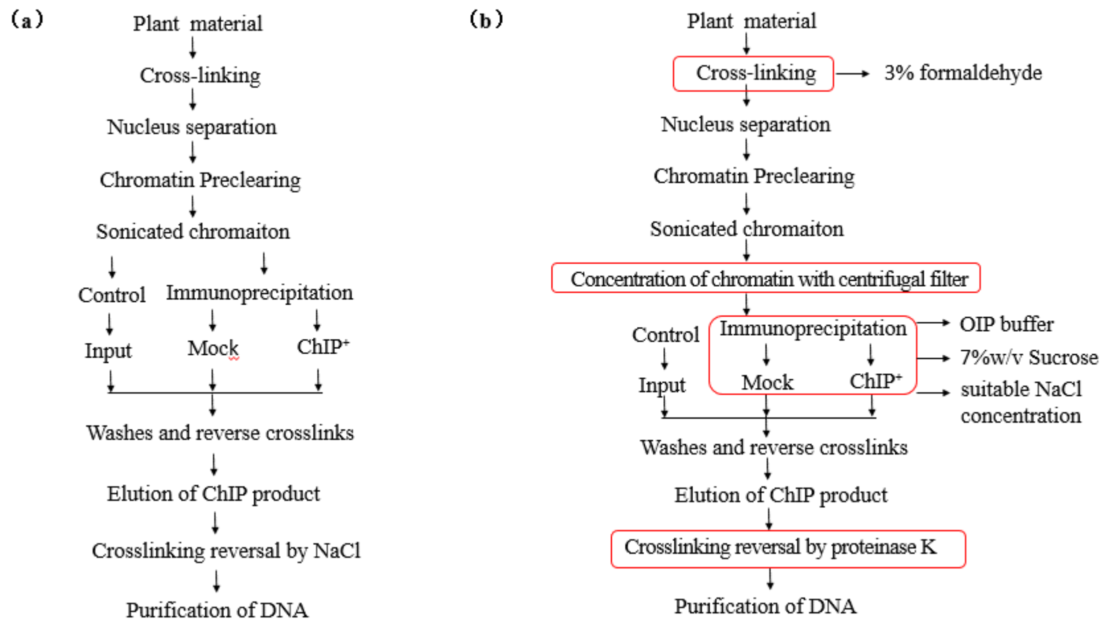

Figure 8. The procedure for the newly developed ChIP protocol.

The outline of the developed ChIP. (a): The procedures for standard ChIP; (b): The procedures for the newly developed ChIP. The optimized procedures are marked with red frames, which included crosslinking protein and chromatin using 3% formaldehyde instead of 1% formaldehyde; purification and concentration of chromatin using protein centrifugal filters; using OIP (optimized immunoprecipitation) buffer instead of standard ChIP Ab incubation buffer for immunoprecipitation; sucrose was added to improve the immunoprecipitation efficiency, and crosslinking reversal was achieved using proteinase K directly. The detailed procedures are shown as supplementary file 1.

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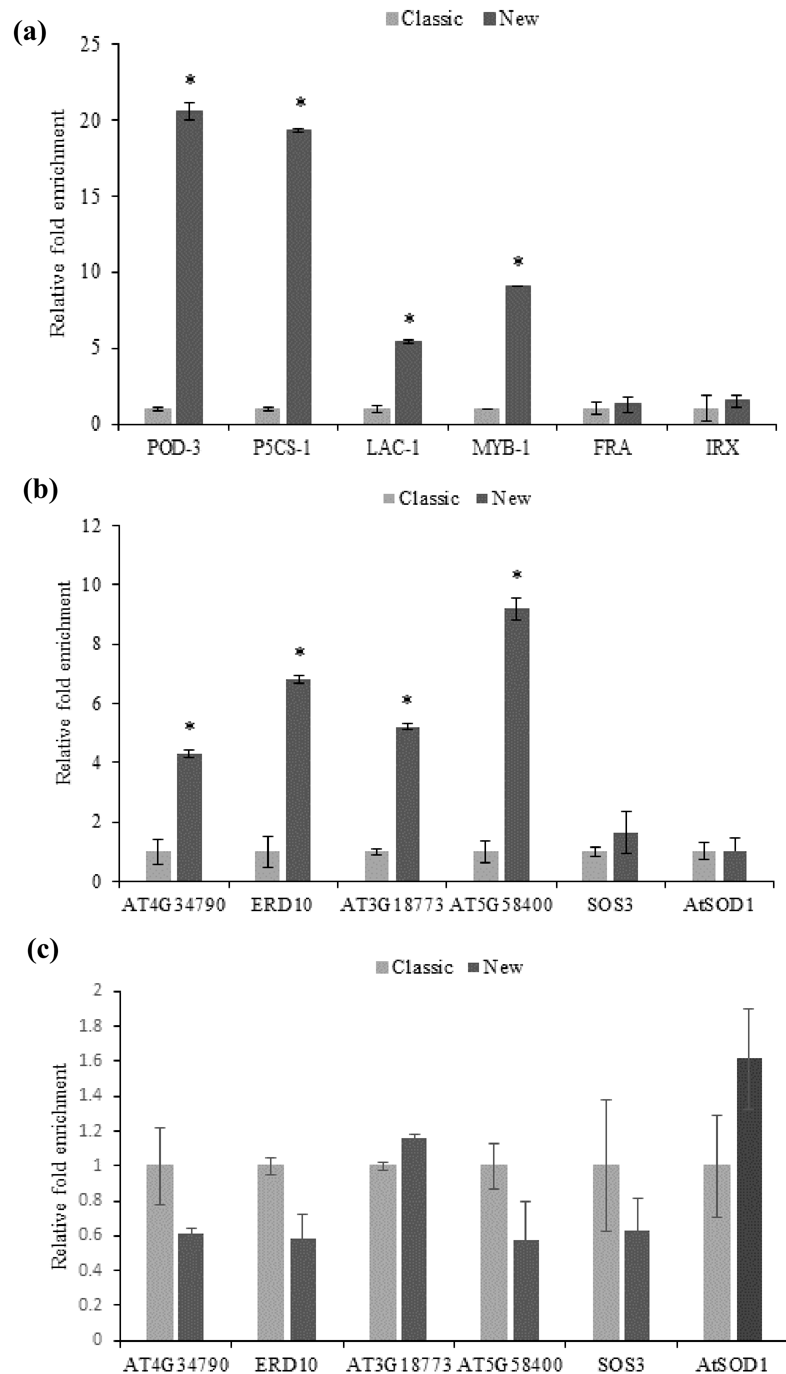

Figure 9. Analysis of the developed ChIP protocol for ChIP enrichment.
ChIP was carried out using the improved ChIP protocol and the standard ChIP protocol. The fold enrichment of ChIP was studied using ChIP-qPCR. Classic: fold enrichment of ChIP performed using the standard ChIP protocol; New: fold enrichment of ChIP performed using the new ChIP protocol. The relatively ChIP fold enrichment were calculated as: New/Classic. (a, b) The ChIP fold enrichment values were compared between the classic and the new ChIP protocol in *B. platyphylla* (a) and *A. thaliana* (b). (c): The Arabidopsis plants overexpression of GFP were used as material for ChIP, and immunoprecipitation was performed with antiGFP antibidy (as negative control). Four truncated promoters of genes from *B. platyphylla* and four truncated promoters of genes from *A. thaliana* were analyzed using ChIP-qPCR. Three independent experiments were performed, and data are means ± SD from three replicates.

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