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A Brassica napus reductase gene dissected by associative transcriptomics enhances plant adaption to freezing stress

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Author contribution statement

Figures, study design: Yong Huang, Muhammad Azhar Hussain;
Investigation and data collection: Yong Huang; Hongzhi Xu; Chuan Zeng;
Data analysis: Guangyuan Lu, Zhitao Tian, LenkaHavlickova, Ian Bancroft;
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Writing-original draft: Yong Huang, Muhammad Azhar Hussain;
Writing-review&editing: Yan Lv, Guangyuan Lu;
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Keywords

alkaloid 3, rapeseed, tropinone reductase, Photosynthetic gas exchange parameter, Associative Transcriptomics

Abstract

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Cold treatment (vernalization) is required for winter crops such as rapeseed (*Brassica napus* L.). However, excessive exposure to low temperature (LT) in winter is also a stress for the semi-winter, early-flowering rapeseed varieties widely cultivated in China. Photosynthetic efficiency is one of the key determinants and thus a good indicator for LT tolerance in plants. So far, the genetic basis underlying photosynthetic efficiency is poorly understood in rapeseed. Here the current study used Associative Transcriptomics to identify genetic loci controlling photosynthetic gas exchange parameters in a diversity panel comprising 123 accessions. A total of 201 significant Single Nucleotide Polymorphisms (SNPs) and 147 Gene Expression Markers (GEMs) were detected, leading to the identification of 22 candidate genes. Of these, Cab026133.1, an orthologue of the Arabidopsis gene AT2G29300.2 encoding a tropinone reductase (BnTR1), was further confirmed to be closely linked to transpiration rate. Ectopic expressing BnTR1 in Arabidopsis plants significantly increased the transpiration rate and enhanced LT tolerance under freezing conditions. Also, a much higher level of alkaloids content was observed in the transgenic Arabidopsis plants, which could help protect against LT stress. Together, the current study showed that AT is an effective approach for dissecting LT tolerance trait in rapeseed and that BnTR1 is a good target gene for the genetic improvement of LT tolerance in plant.

Contribution to the field

The genetic basis underlying the adaptation to unfavourable climate is limited in *Brassica napus*. In the present study, we performed Associative Transcriptomics (AT) analysis on photosynthetic gas exchange parameters in a panel of 123 late-sown *Brassica napus* accessions. Hundreds of candidate CDSs significantly associated with target traits were identified in this study. Then one of the candidate genes encoding tropinone reductase in *Brassica napus* (BnTR1), was functionally validated in Arabidopsis; ectopic expressing BnTR1 could enhance the transpiration rate/ evapotranspiration value and confer freezing tolerance in Arabidopsis plants. To the best of our knowledge, this is the first study to use AT approach for the identification of genes responsible for photosynthetic related traits under chilling stress conditions. We believe that this manuscript would be of interest to the readers of Frontiers in Plant Science, because our findings indicate that AT approach provides an opportunity for genetic dissection of complex traits such as ecological adaptation in crops.

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Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

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Inclusion of identifiable human data

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In review

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Generated Statement: The datasets presented in this study can be found in online repositories. The names of the repository/ repositories and accession number(s) can be found in the article/ supplementary material.

In review

1 **A *Brassica napus* reductase gene dissected by associative transcriptomics**
2 **enhances plant adaption to freezing stress**

3

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18

19 **Abstract:** Cold treatment (vernalization) is required for winter crops such as rapeseed
20 (*Brassica napus* L.). However, excessive exposure to low temperature (LT) in winter
21 is also a stress for the semi-winter, early-flowering rapeseed varieties widely
22 cultivated in China. Photosynthetic efficiency is one of the key determinants and thus
23 a good indicator for LT tolerance in plants. So far, the genetic basis underlying
24 photosynthetic efficiency is poorly understood in rapeseed. Here the current study
25 used Associative Transcriptomics to identify genetic loci controlling photosynthetic
26 gas exchange parameters in a diversity panel comprising 123 accessions. A total of
27 201 significant Single Nucleotide Polymorphisms (SNPs) and 147 Gene Expression
28 Markers (GEMs) were detected, leading to the identification of 22 candidate genes.
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30 encoding a tropinone reductase (*BnTRI*), was further confirmed to be closely linked
31 to transpiration rate. Ectopic expressing *BnTRI* in *Arabidopsis* plants significantly
32 increased the transpiration rate and enhanced LT tolerance under freezing conditions.
33 Also, a much higher level of alkaloids content was observed in the transgenic
34 *Arabidopsis* plants, which could help protect against LT stress. Together, the current
35 study showed that AT is an effective approach for dissecting LT tolerance trait in
36 rapeseed and that *BnTRI* is a good target gene for the genetic improvement of LT
37 tolerance in plant.

38 **Keywords:** Rapeseed, Associative Transcriptomics, photosynthetic gas exchange
39 parameter, tropinone reductase, alkaloid.

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43 **INTRODUCTION**

44 Rapeseed (*Brassica napus* L.) is one of the major oil crops worldwide, with an
45 average annual cropping area of 35.3 million hectares producing 72.8 million tons of
46 seeds in the past five years (<http://www.fao.org/faostat/>). Meal cake, the byproduct of
47 rapeseed is also an important source of protein-rich feed for livestock (Wanasundara
48 et al., 2016). Due to the agronomic importance of this oil crop, there is a great interest
49 to boost its yield via genetic improvement of major agronomic traits.

50 The winter type rapeseed is mainly grown in Europe, which requires strong
51 vernalization and is cold tolerant (O'Neill et al., 2019). However, the semi-winter type
52 rapeseed grown in China only needs moderate or weak vernalization, and excessive
53 exposure to low temperature (LT) stress in winter will lead to plant damage at
54 vegetative stage and finally cause yield loss (Liao and Guan, 2001; O'Neill et al., 2019;
55 Zhang et al., 2015). Yangtze River basin is the major area for growing semi-winter
56 rapeseed, which accounts for at least 80% of the nation's total production (Tian et al.,
57 2018). The rapeseed is usually sown in early October shortly after the harvest of rice
58 in this area (Cong et al., 2019). However, in recent years, the delay of rice harvest
59 usually lead to the postpone of rapeseed sowing until late October or early November,
60 which results in poor germination and seedling establishment due to LT (Luo et al.,
61 2019). The biomass of rapeseed seedling is also significantly reduced at overwintering
62 stage, and thus is more susceptible to LT stresses, i.e. chilling (0–15°C) or freezing
63 (<0°C) (Sage and Kubien, 2007; Zhang et al., 2012; Zhang et al., 2016). Moreover,
64 delay of floral initiation and floral bud differentiation processes (Luo et al., 2018) and
65 decrease of effective pod number, pod length, and seed yield (Ozer, 2003) were
66 observed in the late-sowing rapeseed. To cope with LT stresses, plants have evolved
67 several elaborate regulatory mechanisms; among these, balancing or coordinating the
68 photosynthetic processes could be a critical one (Leister, 2019).

69 It has been established that light-harvesting complex II (LHCII) proteins in
70 higher plants can facilitate their adaption to external biotic or abiotic environmental
71 stresses such as drought stress or blast fungus infection (Andersson et al., 2003;
72 Ganeteg et al., 2004; Liu et al., 2019b; Xu et al., 2012), and the positive function of

73 LHCBS in abscisic acid (ABA) mediated signalling pathway is repressed by
74 WARY40 (Liu et al., 2013; Xu et al., 2012). LT stress induces the accumulation of
75 transcript encoding heat-shock proteins (HSPs), and the persistence of HSPs can
76 enhance the chilling tolerance of tomato fruit (Ding et al., 2001; Sabehat et al., 1996).
77 In *Arabidopsis*, HSP21 protects the photosynthetic electron transport chain against the
78 deleterious effects imposed by heat stress (Bernfur et al., 2017; Zhong et al., 2013).
79 However, it is unclear whether HSPs function similarly in rapeseed to alleviate injury
80 from LT stress. The other observations have mechanistically described how the
81 photosynthetic organisms maintain their PSII function under stressful conditions with
82 continuing or fluctuating light (Liu et al., 2019a). For instance, the chloroplast protein
83 HHL1 forms a complex with LQY1 to repair and re-assemble PSII, which in turn
84 helps overcome excessive light stress (Jin et al., 2014). In rapeseed, CBF/DREB
85 transcription factors appear to have important roles in maintaining stronger
86 photosynthetic efficiency and higher Calvin circulating enzyme activity under LT
87 conditions (Savitch et al., 2005).

88 To date, several genetic studies have been reported for quantitative trait loci
89 (QTLs) mapping of photosynthesis (Basu et al., 2019; De Miguel et al., 2014; Li et al.,
90 2014; Li et al., 2016; Liu et al., 2017; Oakley et al., 2018) and LT tolerance in
91 different plant species (Jha et al., 2017). However, very few QTLs have been
92 identified in *Brassica* species (Ge et al., 2012; Yan et al., 2015). The temperature
93 causing 50% of the maximal damage (LT50) has been regarded as a good index for
94 evaluating LT tolerance (Hincha et al., 1987; Steponkus et al., 1990), which is also
95 significantly correlated with net photosynthesis rate (A_n) in rapeseed (Urban et al.,
96 2013). Therefore, photosynthetic gas exchange parameters such as A_n are a suitable
97 index for the evaluation of LT tolerance in rapeseed that can facilitate the follow-up
98 genetic study.

99 Photosynthesis plays an indispensable role in ensuring adequate energy supply
100 throughout the plant lifecycle. Therefore, enhancing photosynthetic efficiency has
101 been a commonly adopted strategy for crop yield improvement (Evans, 2013; Lawson
102 et al., 2012; Long et al., 2006). In rice, a new photorespiratory bypass was assembled

103 by over-expressing *OsGLO3*, *OsOXO3* and *OsCATC* genes in the chloroplast, which
104 resulted in obvious increases in photosynthetic efficiency, biomass and grain yield
105 (Shen et al., 2019). In rapeseed, photosynthetic efficiency has a notable effect on yield,
106 oil content and fatty acid composition (Ju and Li, 2012; Wang et al., 2015). However,
107 the utilization rate of light energy in rapeseed is only 0.615-1.056%, which is much
108 lower than that in rice, wheat or soybean (Zhang et al., 2017a). Therefore, it is
109 possible to further improve the yield by enhancing photosynthetic efficiency in
110 rapeseed.

111 During the long history of evolution, plants appear to overcome abrupt or mild
112 temperature stresses in winter through a series of changes at molecular, cellular,
113 physiological and biochemical levels (Zhang et al., 2017b). Alkaloid is one of the
114 major secondary metabolites that is inducible under unfavourable conditions,
115 especially drought stress (Selmar and Kleinwachter, 2013). Heavy metals can also
116 promote the accumulation of alkaloid in *Catharanthus roseus* L. (Srivastava and
117 Srivastava, 2010). The short-chain dehydrogenase/reductase (SDR) protein, which
118 belongs to the NAD(P)-binding Rossmann-fold superfamily, functions in the
119 biosynthesis of benzyloquinoline alkaloids (i.e. morphine, codeine) and
120 tropane-derived alkaloids such as scopolamine, atropine and cocaine. Tropane
121 reductases (TRs) are a group of SDR proteins which play key roles as a branch point
122 in the biosynthesis pathway of tropane alkaloids (Tonfack et al., 2011). Hence, a
123 study on TRs could help understand how alkaloids function in response to different
124 stresses.

125 Associative Transcriptomics (AT) strategy, which combines association mapping
126 and transcriptome, has greatly facilitated the genetic dissection of complex traits
127 (Bazakos et al., 2017). Considerable progress has been achieved by AT in
128 allopolyploidy crops such as oilseed rape and wheat, which provided a large number
129 of causative genes or functional markers for molecular marker-assisted breeding. For
130 instance, a transcription factor (HAG1) was identified in rapeseed by AT, which plays
131 an indispensable role in the synthesis of aliphatic glucosinolates (Harper et al., 2012).
132 With AT platform, the genetic studies of many other complex traits in rapeseed were

133 also reported, including homeostasis of nitrate, phosphate and sulfate anions
134 (Koprivova et al., 2014), calcium and magnesium accumulation (Alcock et al., 2017),
135 lodging resistance (Miller et al., 2018), clubroot resistance (Hejna et al., 2019), erucic
136 acid and tocopherol (vitamin E) isoform accumulation in seeds (Havlickova et al.,
137 2018), and leaf nutrition concentration (Alcock et al., 2018). Recently, the power of
138 AT was further enhanced by using a much larger panel comprising 383 rapeseed
139 accessions (Havlickova et al., 2018). In bread wheat, two causative genes underlying
140 stem strength variation have also been detected by AT (Miller et al., 2016). Despite
141 the above efforts, the AT approach has not yet been applied to photosynthetic related
142 traits under LT conditions in rapeseed.

143 The present study aims to identify candidate genes associated with
144 photosynthetic gas exchange parameters including A_n , stomatal conductance to water
145 vapour (G_{sw}), internal CO_2 concentration (C_i) and transpiration
146 rate/evapotranspiration (E) in rapeseed by AT. Twenty-two candidate genes were
147 obtained, and one was functionally validated. Ectopic expressing tropinone reductase
148 (*BnTR1*) in *Arabidopsis* can significantly enhance transpiration rate and LT tolerance,
149 implying its great potential for the genetic improvement of LT tolerance in plant.

150

151 **MATERIALS AND METHODS**

152 **Plant materials**

153 A panel comprising 123 rapeseed accessions was used for association study,
154 which is available from the John Innes Centre, Norwich, UK (**Supplementary Table**
155 **S1**) (Havlickova et al., 2018). Within this panel, there are 37 winter type, 32 spring
156 type, 47 semi-winter type and 7 unclassified rapeseed accessions. The panel was sown
157 on 28th Oct in 2016 in Wuhan (114.30°E, 30.57°N), China. All accessions were
158 planted using a completely randomized block design with three replications. The
159 temperature was recorded during the field experiments (**Supplementary Figure S1**).
160 Compared with those sown on normal occasion (28th Sept in 2016), the late-sown (i.e.
161 28th Oct) rapeseed seedlings were subjected to LT stress during winter.

162 **Determination of photosynthetic gas exchange parameters**

163 The fourth true leaf of each of the 123 rapeseed accessions was chosen for the
164 measurement of photosynthetic gas exchange parameters including A_n , G_{sw} , C_i and E
165 in the open field at the 60-d-old seedling stage. Two independent plants of one
166 accession from each block were measured by LI-6400 photosynthesis equipment
167 (Li-Cor 6400, Li-CorInc, Lincoln, NE, USA) as described previously (Yan et al.,
168 2015). The measurements were performed on a sunny day from 27th December to 30th
169 December with a maximum temperature of 9°C in daytime and the lowest
170 temperature of -1°C at night. The phenotypic data were collected from three blocks as
171 biological replications (**Supplementary Table S2**). Broad-sense heritability was
172 estimated according to a previous study (Kaler and Purcell, 2019).

173 The photosynthetic gas exchange parameters of *Arabidopsis* wild type (WT) and
174 transgenic seedlings (ecotype Columbia) were measured on the second functional leaf
175 with the light intensity of 800 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and CO_2 concentration of 400 $\mu\text{L L}^{-1}$. All of
176 the *Arabidopsis* plants were grown in the greenhouse (16-h-light/8-h-dark) with the
177 light intensity of 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at 23°C. Each leaf was measured three times as
178 technical replications, and five independent plants from each line were measured as
179 biological replications (**Supplementary Table S2**).

180 **Genome-wide association study**

181 The association panel and the procedure of AT analysis have been reported in
182 detail previously (Havlickova et al., 2018). In brief, RNA-Seq data were generated
183 from young leaves of the association panel harvested 21 d after sowing under
184 16-h-light (20°C)/8-h-dark (14°C) glasshouse conditions. The transcriptome data were
185 mapped onto the developed ordered *Brassica* A and C pan-transcriptomes (He et al.,
186 2015) and resulted in a set of 355,536 SNPs and RPKM values for 116,098 CDS
187 models. Following the removal of SNP markers with minor allele frequencies below
188 0.01, a total of 256,397 SNPs were retained (<http://www.yorkknowledgebase.info/>) and
189 used as marker input for the Associative Transcriptomics analysis as previously
190 described (Harper et al., 2012; Lu et al., 2014). The current study adopted a
191 compressed mixed linear model including both fixed and random effects according to
192 a previous method (Lipka et al., 2012). The P -values ($-\log_{10}$ converted) for all SNPs

193 were plotted against their physical position in the ‘pseudo-molecules’ to produce a
194 Manhattan plot. The Bonferroni significance threshold was set as $P=3.9 \times 10^{-6}$
195 ($1/256397$) (Duggal et al., 2008). Allelic effects of all candidate SNPs were calculated
196 according to a previous study (Prado et al., 2017); a positive effect indicates that the
197 allele increases the trait value, whereas a negative effect indicates that the alternative
198 allele increases the trait value.

199 The transcript level was quantified as reads per kb per million aligned reads
200 (RPKM) across the panel. After filtering ($RPKM \leq 0.4$), a total of 53,889 gene
201 expression marker (GEM) was obtained. The GEM was regarded as the dependent
202 variable and trait data as the independent variable (Wood et al., 2017). The fixed
203 effect linear model was performed to assess the relationship between gene expression
204 level and the traits (Alcock et al., 2017). The P -value for each GEM was converted
205 ($-\log_{10}P$) and plotted against its physical position to generate a Manhattan plot. The
206 Bonferroni significance threshold was set at $P=1.85 \times 10^{-5}$ ($1/53889$).

207 **Growth conditions and stress treatments**

208 To determine the gene function of *BnTR1* on *Arabidopsis* under freezing
209 conditions, the seeds of WT and transgenic *Arabidopsis* plants were germinated on
210 1/2 MS medium and 1/2 MS medium plus 30 $\mu\text{g}/\text{mL}$ hygromycin, respectively. After
211 1 week growth at 23°C (16-h-light/8-h-dark), healthy seedlings with uniform sizes
212 were transplanted into 8×8 cm pots (four plants per pot). Then, the 21-d-old plants
213 were transferred into a growth chamber at -4°C for 4 h after 24 h of cold acclimation
214 and recover at 23°C (16-h-light/8-h-dark). Six pots from each transgenic line or WT
215 plants were used to investigate the survival rate 3 d after recovery. The leaves were
216 sampled for physiological and biochemical measurements.

217 To determine the effects of alkaloid on *Arabidopsis* and rapeseed seedlings, WT
218 plants of *Arabidopsis* were firstly grown under the same conditions as above. Before
219 the freezing treatment, a dosage of 0, 10, and 30 nmol alkaloid (atropine) per seedling
220 was added to the soil for *Arabidopsis*, while a dosage of 0, 50 and 150 nmol per
221 seedling was added to the soil for rapeseed accession Zhongshuang 11 (ZS11)
222 according to the previous study (Hara and Kurita, 2014). After inoculation overnight,

223 the 21-d-old *Arabidopsis* plants were transferred into a growth chamber at -4°C for 4
224 h, the three-leaf-stage rapeseed plants were transferred into the growth chamber at
225 -4°C for 4 h (Yan et al., 2019). The survival rate was investigated in six pots 3 d after
226 recovery (**Supplementary Table S2**).

227 To investigate the expression of candidate genes under LT stress conditions, six
228 rapeseed accessions showing extreme photosynthetic efficiency (i.e. Sv706118, Kajsa,
229 Callypso, Libritta, Gefion and Jupiter; **Supplementary Table S1**) were used for
230 expression analysis of candidate genes. The four-leaf seedlings were transferred into
231 the plant growth chamber with -4°C for 4 h (Yan et al., 2019). The leaves were
232 sampled before, and after freezing treatment, and then used for RNA extraction and
233 molecular analysis. The detailed information was listed in **Supplementary Table S2**.

234 **Physiological and Biochemical Measurements**

235 To determine the effects of *BnTR1* on *Arabidopsis* at physiological and
236 biochemical levels under LT stress conditions, the seedlings of transgenic and WT
237 *Arabidopsis* plants were treated under freezing conditions (-4°C for 4 h) and, the
238 leaves of 21-d-old seedlings were sampled at three time-point (i.e. before freezing
239 treatment, after freezing treatment, and recovery for 3 d at 23°C) for measuring
240 physiological and biochemical characteristics, including Fv/Fm, electrolyte leakage,
241 3,3'-diaminobenzidine (DAB) staining, proline content, soluble sugar content,
242 reactive oxygen species (ROS) scavenging enzymes activity, H₂O₂ content, alkaloid
243 content. All measurements were performed with at least three biological replications;
244 detail information for experimental design and plant materials used was listed in
245 **Supplementary Table S2**.

246 The Fv/Fm measurement was performed using the second functional leaves
247 before and after the freezing treatment. The leaves were firstly immersed in 1%
248 agarose overnight avoiding of the dark, then the chlorophyll measurement (Fv/Fm)
249 was measured using the modulated chlorophyll fluorescence instrument (PAM-2500;
250 Walz) as previously reported (Lv et al., 2017).

251 The electrolyte leakage measurement was performed according to the previous
252 study (Lv et al., 2016). Briefly, six leaves from six were cut and immersed in 8 mL of

253 double-distilled H₂O in a 10-mL tube. After shaking overnight, the electrolyte leakage
254 was measured using a model DDS-IIA device (Leici Instrument) as R1; it was
255 measured again and recorded as R2 after boiling at 95°C in a water bath for 15 min
256 and cooling down. The relative electrolyte leakage was calculated as a ratio of R1/
257 R2.

258 The proline and soluble sugar contents were measured using the kits from Beijing
259 Solarbio Science & Technology as described before (Yan et al., 2019). In brief, 0.1 g
260 fresh tissue was powdered and incubated in 1 ml 3% sulfosalicylic acid (for proline)
261 or ddH₂O (for soluble sugar). After centrifuging, 400 µL supernatant was mix with
262 other reaction buffers and incubated at 95°C in a water bath for 15 min, then the
263 absorbance was measured using MULTISCAN FC (Thermo Scientific).

264 The ROS scavenging enzymes activity was measured by commercial kits
265 according to the manufacturer's instruction (Beijing Solarbio Science & Technology)
266 with minor modification (Yan et al., 2019). 0.1 g fresh tissue was powdered using 1
267 mL 0.05 mol/L PBS buffer (pH 7.8). The supernatant was obtained after centrifuging
268 at 8,000 g for 10 min at 4°C and used for superoxide dismutase (SOD) activity,
269 peroxidase (POD) activity, catalase (CAT) activity measurement using MULTISCAN
270 FC (Thermo Scientific).

271 DAB staining was performed as previously described (Ning et al., 2010; Zhang
272 et al., 2011). The fourth functional leaf of each plant was sampled and infiltrated in
273 0.1 mg/mL 3,3'-diaminobenzidine liquid (50 mM Tris-acetate buffer, pH 5.0). After
274 incubation overnight at 25°C in the dark, the stained leaves were photographed after
275 removing the chlorophyll by absolute ethanol. The H₂O₂ content was quantified
276 according to the instruction of the kit (Beijing Solarbio Science & Technology) (Yan
277 et al., 2019).

278 The total alkaloid was extracted as described previously (Chen et al., 2013;
279 Zhang et al., 2004). The freeze-dried leaves were powdered; 0.1 g powder was
280 homogenized overnight with 1.0 mL 70% aqueous methanol at 4°C. Following
281 centrifugation at 10,000 g for 10 min at 4°C, the extracts were absorbed (CNWBOND
282 Carbon-GCB SPE Cartridge, 250 mg, 3 mL; ANPEL) and filtrated (SCAA-104, 0.22

283 μm pore size; ANPEL). Next, the total alkaloid content was determined using the
284 alkaloid ELISA kit (Hiton) according to the instructions.

285 **RNA extraction and gene expression analyses**

286 Total RNA was extracted from the *Arabidopsis* or rapeseed seedlings with
287 TransZol reagent (Trans) and converted to the first-strand cDNA using the
288 EasyScript®One-Step cDNA Synthesis SuperMix (Trans). The Quantitative Real-time
289 PCR (qRT-PCR) was performed using Power SYBR®Green PCR Master Mix
290 according to the manufacturer's instructions on a StepOnePlusReal-Time PCR
291 System (Applied Biosystems). The primers used for expression analysis of candidate
292 genes either detected by AT approach or involved in the known biosynthetic pathway
293 of alkaloid were listed in **Supplementary Table S3**. The relative expression level was
294 determined as previously described (Livak and Schmittgen, 2001).

295 **Vector construction and gene transformation**

296 To generate transgenic lines over-expressing candidate gene (*BnTR1*), the coding
297 sequence (CDS) of *LOC106445422* was amplified from a rapeseed variety ZS11 and
298 ligated into vector pCAMBIA1300 driven by a tobacco cauliflower mosaic virus 35S
299 promoter (CaMV35S). The construct was introduced into *Arabidopsis* variety
300 Columbia (Col) by *Agrobacterium*-mediated transformation (Zhang et al., 2006).
301 Three T4 homozygous lines (L1, L3, L5) significantly over-expressing *BnTR1* were
302 obtained by screening at 30 $\mu\text{g}/\text{mL}$ hygromycin. The primers used for vector
303 construction were listed in **Supplementary Table S3**.

304 **Statistical analyses**

305 Statistical analyses were conducted with Microsoft Excel (2003) and SPSS
306 (version 22.0) software using one-way analysis of variance (ANOVA) with Tukey's
307 multiple comparisons test or two-tailed Student's *t*-test. All data were presented as the
308 means \pm standard error (SE) based on three replicates. $P < 0.05$ and $P < 0.01$ were
309 considered statistically significant and highly significant, respectively.

310

311 **RESULTS**

312 **Phenotypic variation of photosynthetic gas exchange parameters**

313 A field-grown rapeseed association panel experienced long-term LT stress in
314 winter (**Supplementary Figure S1**). The photosynthetic gas exchange parameters
315 such as A_n , G_{sw} , C_i and E were measured since they can reflect photosynthetic
316 efficiency for a plant. Substantial variations for the four traits were observed in 123
317 rapeseed accessions (**Figure 1**). A_n varied from 12.87 to 24.02 with a mean of 19.29
318 $\mu\text{mol (CO}_2\text{) m}^{-2}\text{s}^{-1}$. Similarly, G_{sw} ranged from 245.21 to 383.28 $\mu\text{mol (CO}_2\text{) mol}^{-1}$
319 and E from 0.96 to 3.75 $\text{mmol (H}_2\text{O) m}^{-2}\text{s}^{-1}$. G_{sw} was the most variable trait since it
320 has the largest coefficient of variation (0.69), with a minimum of 0.16 and a
321 maximum of 2.53 $\text{mol (H}_2\text{O) m}^{-2}\text{s}^{-1}$; the range of broad-sense heritability varied from
322 49.49% to 68.91% (**Supplementary Table S4**). Moreover, the four traits in the
323 association panel were positively correlated with each other ($P \leq 0.01$)
324 (**Supplementary Table S5**). For instance, there was a strong correlation between A_n
325 and E ($r = 0.785$, $P < 0.01$).

326

327 **Associative Transcriptomics for photosynthetic gas exchange parameters**

328 To identify genomic regions controlling photosynthetic related traits, AT was
329 performed in rapeseed. Using the mixed linear model, a total of 201 significant SNPs
330 were detected, which originated from 148 CDSs (**Figure 2**). Unexpectedly, most of
331 the significant CDSs were associated with G_{sw} trait, while only one was related to E
332 trait. The detail results including physical positions, P -values, allelic effects were
333 summarized in **Supplementary Table S6**. For GEM analysis, a total of 145 CDSs
334 above the corrected Bonferroni thresholds were identified (**Figure 2**). Of these, a
335 respective of 5, 10, 20 and 110 CDSs were detected for A_n , G_{sw} , C_i and E . The detail
336 information including physical positions and P -values for all significant GEMs was
337 listed in **Supplementary Table S7**.

338 By annotating all the above significant CDS in public database TAIR
339 (<https://www.arabidopsis.org/>), the present study shortlists the number of candidate
340 genes to only 22 that putatively involved in photosynthesis or LT stress response
341 (**Supplementary Table S8**). To verify their roles in rapeseed with or without LT
342 stress, six accessions from the association panel were selected; Sv706118, Kajsa, and

343 Callypso (Accessions 1-3) exhibited higher photosynthesis efficiency and were
344 tolerant to freezing stress, while Libritta, Gefion, and Jupiter (Accessions 4-6)
345 presented lower photosynthesis efficiency and were sensitive to freezing stress
346 (**Supplementary Table S1, Supplementary Figure S2**). The gene expression
347 profiles were investigated by qRT-PCR in the six accessions under freezing
348 conditions. Results showed that all of these genes exhibited a significantly different
349 expression level in the six accessions, suggesting that these genes indeed involved in
350 freezing stress response. *Cab026133.1* seemed to have a much higher expression level
351 in the three tolerant accessions (Accessions 1-3) with or without freezing stress
352 (**Figure 3A**); so did *Cab011968.1*, *Cab022014.2*, and *Cab007526.2*, an orthologue of
353 inorganic carbon transport protein (AT1G70760.1), bZIP transcription factor
354 (AT5G28770.3) and citrate synthase 2 (BnaA10g24440D), respectively. An opposite
355 trend was observed for *Bo5g017460.1* and *Cab008128.1*, an orthologue of F-box
356 family protein (AT2G32560.1) and dehydroascorbate reductase (AT5G16710.1),
357 respectively (**Supplementary Figure S3**).

358

359 **Selection and characterization of candidate gene**

360 *Cab026133.1* was selected for further analysis because it not only exhibited the
361 highest *P*-value (6.33×10^{-9}) for E trait in GEM analysis (**Supplementary Table S8**)
362 but also highly expressed in LT tolerant accessions (**Figure 3A**). Besides, the
363 expression of *Cab026133.1* (presented as RPKM) across the rapeseed panel was
364 positively correlated with E level ($r=0.406$, $P < 10^{-3}$) and accounted for 16.5% of trait
365 variation (**Figure 3B**). The ortholog of *Cab026133.1* in *Arabidopsis* (AT2g29300)
366 encodes an SDR protein involved in the oxidation-reduction process of secondary
367 metabolites such as phenols, isoprene and alkaloid (Selmar and Kleinwachter, 2013).
368 SDR proteins are classified into six subfamilies, and the tropinone reductase
369 subfamily belongs to the major route of alkaloid biosynthesis (Tonfack et al., 2011).
370 The alignment of amino acid sequence clearly illustrated that LOC106445422 shared
371 87.3% similarity with *Cab026133.1* and 57% with CoTR, a known tropinone
372 reductase in *Cochlearia officinalis* (Brock et al., 2008). LOC106445422 displayed

373 typical SDRs motifs (Gly-X₃-Gly-X-Gly) and four conserved residues that form the
374 catalytic tetrad NSYK (N127, S155, Y168, K172) (**Supplementary Figure S4**). The
375 current study name LOC106445422 as *BnTR1* and used as the candidate gene for the
376 follow-up studies.

377 To assess the effect of allelic variation on *BnTR1* in the rapeseed association
378 panel, the genomic region covering the whole gene as well as the 2-kb promoter
379 region was amplified. One TAA/TAATAA insertion was detected in the fourth intron
380 that formed two major haplotypes, i.e. Haplotype I (with TAA insertion) and
381 Haplotype II (with TAATAA insertion) (**Figure 3C**). Haplotype I (n=31) displayed
382 significantly higher E value than Haplotype II (n=60) ($P=5.43\times 10^{-4}$) (**Figure 3D**).
383 Besides, the LT tolerant Accessions 1-3 were determined as Haplotype I while
384 sensitive Accessions 4-6 as Haplotype II at *BnTR1* locus (**Supplementary Table S1**).
385 The expression level of *BnTR1* in Accessions 1-3 was also significantly higher than
386 that in accessions 4-6 with or without stress treatment (**Figure 3A**). Therefore, it was
387 evident that E variation may be attributed to expression or allelic variation at *BnTR1*,
388 which was expressed almost in all tissues of rapeseed at both vegetative and
389 reproductive stages (**Supplementary Figure S5**).

390

391 **Ecotopic expressing *BnTR1* enhances freezing tolerance in *Arabidopsis***

392 To analyze gene function, three independent *Arabidopsis* lines (L1, L3, L5)
393 ectopic expressing *BnTR1* were generated. All transgenic lines showed an increased
394 expression level of *BnTR1* in comparison to the WT plants (**Figure 4A**). At the
395 seedling stage, E value of the transgenic lines was much higher than that of WT plants
396 (**Figure 4B**), thus confirming that *BnTR1* controls transpiration rate. Since
397 photosynthetic gas exchange parameters are significantly correlated with cold
398 tolerance (Urban et al., 2013), it was speculated that BnTR1 also involves in LT stress.
399 To test this hypothesis, seedlings were treated at -4°C for 4 h and then recover at 23°C.
400 All of the transgenic lines were shown to be freezing-tolerant because there was no
401 obvious syndrome, while the WT plants were wrinkled and hydrophanous (**Figure**
402 **4C**). After recovery for 3 d at 23°C, all transgenic plants survived, but 62% of the WT

403 plants died (**Figure 4D**). These results strongly suggested that *BnTR1* enhanced the
404 freezing tolerance of *Arabidopsis* plants.

405 To assess whether C-repeat-binding factors (CBFs) contributed to the enhanced
406 freezing tolerance of the transgenic lines, the current study measured the relative
407 expression level of *CBFs* in transgenic and WT plants after freezing stress (**Figure**
408 **4E-G**). In the transgenic lines grown under normal conditions, expressions of *CBFs*
409 were much higher than that in WT plants, indicating that they were markedly induced
410 by *BnTR1*. Moreover, the expression of *CBF1* and *CBF3* was significantly
411 up-regulated and *CBF2* down-regulated by *BnTR1* during freezing treatment in
412 comparison with those of normal conditions. The current study further examined the
413 expression level of CBFs-targeted cold-responsive genes (COR genes) (**Figure 4H-I**).
414 As expected, the transcript levels of COR genes (namely *COR15* and *RD29A*) in the
415 transgenic lines were highly induced when compared with that in WT plants. These
416 results indicated that *BnTR1* influences CBF regulon in the stress-signalling pathway
417 to control freezing tolerance.

418 The chlorophyll fluorescence parameter *Fv/Fm*, an indicator for the potential
419 maximum photosystem II (PSII) capacity of plants, has been widely used to determine
420 the ability of tolerance to environmental stresses under laboratory conditions (Mishra
421 et al., 2014; Thalhammer et al., 2014). Here, markedly higher *Fv/Fm* ratio was
422 observed in leaves of the transgenic lines under freezing as well as normal conditions
423 (**Figure 5A**). To further clarify whether the difference of photosynthetic capacity was
424 caused by the excessive expression of *BnTR1*, the current study assessed the
425 expression level of *BnTR1* and other genes involved in photosynthetic processes such
426 as *RCA*, *SBPASE* (for CO₂ fixation or assimilation) and *CABI-4* (for light-harvesting)
427 (Basu et al., 2019; Sun et al., 2017) (**Figure 5B-H**). Under normal conditions, the
428 expression level of *RCA*, *SPASE* and *CABI* were slightly reduced in the transgenic
429 lines compared to WT plants, whereas *CAB2*, *CAB3* and *CAB4* were induced. The
430 freezing treatment led to a notable suppression of all genes; however, the expression
431 of genes in the transgenic lines returned to a high level when freezing stress was
432 removed.

433

434 **BnTR1 contributes to cell membrane protection and antioxidants**

435 Altering the osmotic balance to maintain the integrity and stability of cell
436 membrane is proposed to be an efficient way for plants adapting to the changing
437 environments (Morsy et al., 2005; Valerio et al., 2011). To test this hypothesis,
438 physiological and biochemical assays were carried out. Results showed that freezing
439 treatment led to only 40-200% increase of proline content in WT plants but as high as
440 100-300% in transgenic plants, indicating that the transgenic plants expressing *BnTR1*
441 could accumulate more proline (**Figure 6A**). The soluble sugar content showed a
442 similar pattern (**Figure 6B**). However, the electrolyte leakage increased more rapidly
443 in WT plants than in transgenic lines (**Figure 6C**). These results implied that BnTR1
444 actively responded to freezing stress by maintaining cell membrane stability and
445 osmotic balance.

446 Antioxidants, which function in scavenging the reactive oxygen species (ROS),
447 are generally considered as another effective element in defending abiotic stresses
448 (Choudhury et al., 2017). To determine whether *BnTR1* affects the antioxidant system,
449 the accumulation of ROS was determined by DAB staining. The brown precipitate
450 (H_2O_2) in WT was much larger than that in the transgenic lines (**Figure 6D**),
451 indicating that WT plants had a higher level of H_2O_2 content than transgenic plants
452 (**Figure 6E**). Oxidoreductases like POD, SOD and CAT also function in scavenging
453 redundant ROS (Gupta et al., 2016). Here, we found that the transgenic lines
454 exhibited stronger SOD activity than WT (**Figure 6F**), which help plants alleviate
455 oxidation damage from freezing conditions. However, no significant difference was
456 detected for POD and CAT activities. Together, the enhanced freezing adaption for
457 transgenic plants could be attributed to the increased ROS scavenging ability.

458

459 **BnTR1 positively affects alkaloid metabolism**

460 BnTR1 (homolog of AT2g29300) is predicted to be a tropinone reductase
461 involved in the biosynthesis of alkaloid (KO00960), which mainly produces atropine.
462 Although all alkaloids (with more than 12,000 different structures) have been

463 well-documented in pharmacology, their roles in abiotic stress remain elusive
464 (Schlager and Drager, 2016). To investigate the specific role of BnTR1 in alkaloid
465 metabolism, the total alkaloids content was quantified. As expected, the transgenic
466 lines led to one- to two-fold increase of alkaloids contents compared with WT plants
467 after freezing stress (**Figure 7A**). To further confirm the effect of alkaloid on stressed
468 plants, exogenous atropine was applied to WT plants, since atropine was considered
469 to be the product of alkaloid metabolism (KO00960) (Hara and Kurita, 2014). Results
470 demonstrated that application of 10 nmol atropine per plant significantly rescued the
471 susceptibility of WT plants, but the protective effect was weakened when dosage
472 increase to 30 nmol (**Figure 7B**). The survival rate increased by three- to four-fold
473 compared with WT plants without atropine treatment (**Figure 7C**).

474 To further elucidate the protective role of alkaloid in rapeseed, the current study
475 applied exogenous atropine to a widely cultivated rapeseed variety, ZS11, under
476 freezing conditions. Phenotypic analysis showed that the wilting phenotype of ZS11
477 plants was partially rescued by exogenous atropine application (50 and 150 nmol per
478 plant) (**Figure 7D**), while no significant difference was observed in the survival rate.
479 It was concluded that alkaloids alleviated the damage on plants from extreme LT
480 stress.

481

482 **DISCUSSION**

483 **Power of AT approach**

484 *Brassica napus* originated from the hybridization of *Brassica rapa* and *Brassica*
485 *oleracea* which contribute the A and C genomes, respectively (Cheung et al., 2009).
486 There is only 15% difference in nucleotide structure and 3% difference in
487 transcriptional expression patterns between chromosomes A and C, which limits the
488 development of SNP markers in genome-wide association analysis until the
489 availability of the high throughput next-generation sequencing technology (Adams et
490 al., 2003; Higgins et al., 2012). Over the past years, AT approach based on abundant
491 SNP markers and GEMs, has successfully simplified the complexity of the whole
492 genome (Harper et al., 2012), and has been widely applied in rapeseed, wheat and

493 other polyploidy crops (Alcock et al., 2017; Harper et al., 2016a; Harper et al., 2016b;
494 Harper et al., 2012; Havlickova et al., 2018; Koprivova et al., 2014; Lu et al., 2014;
495 Miller et al., 2018; Miller et al., 2016; Schuster et al., 2013). However, the genetic
496 basis of photosynthetic-related traits in oil crops remains elusive. Here, the genetic
497 architecture of photosynthetic gas exchange parameters was investigated by AT
498 approach, and a gene termed *BnTR1* was confirmed to be responsible for E trait
499 (Figure 4), which might be a promising candidate beneficial to rapeseed in coping
500 with climatic changes. Several other interesting candidates were also identified. For
501 instance, Bo5g155110.1 was found to be significantly associated with E traits
502 ($P=1.1\times 10^{-5}$), and down-regulated by freezing stress (**Supplementary Figure S3**).
503 The homolog in *Arabidopsis* is Cyclophilin38 (*AtCYP38*), which functions in the
504 assembly and maintenance of PSII super complex (**Supplementary Table S7**). The
505 loss-of-function mutant of *AtCYP38* shows reduced growth rate and photosynthetic
506 efficiency compared to its wild type. Additionally, the D1 and D2 proteins in PSII
507 reaction centre show a short half-life, resulting in susceptibility upon exposure to
508 excessive light (Fu et al., 2007; Sirpio et al., 2008). Bo3g153100.1 was hit by an SNP
509 marker, with $-\log_{10}$ (P -value) value as high as 9.06 (**Supplementary Table S6**), it
510 was also markedly up-regulated by freezing stress (**Supplementary Figure S3**).
511 Bo3g153100.1 was homologues to AT4G37930 in *Arabidopsis*, which has been
512 documented in the photorespiration process (Takahashi et al., 2007). In the knockout
513 mutant of AT4G37930, the photorespiration pathway is destroyed, and the
514 chlorophyll deficiency results in chlorosis (Voll et al., 2006). Therefore, it seems that
515 AT is a powerful tool to identify candidate genes for photosynthesis and LT stress in
516 rapeseed. It is worthy to further study the function of all 22 candidate genes identified
517 here.

518

519 **The positive role of *BnTR1* under LT conditions**

520 It is generally accepted that photosynthesis is vulnerable to adverse
521 environmental stresses such as extreme temperature, salinity, drought or combined
522 stresses (Sainz et al., 2010; Strzepek et al., 2019). Abiotic stresses lead to

523 photoinhibition as well as excessive generation of ROS, which suppresses the
524 photosynthetic progress and ultimately repress the growth and productivity in plants
525 (Gabriel et al., 2010; Nishiyama et al., 2014). During the long-term evolution, plants
526 have developed a variety of adaptive mechanisms to cope with the stressful conditions
527 (Liu et al., 2019a; Strzepak et al., 2019). The CBF transcription factors in rapeseed
528 are known to be responsible for the photosynthetic performance; CBF5 and CBF17
529 enhance the energy conversion efficiency under LT conditions (Dahal et al., 2012;
530 Savitch et al., 2005). CBF1-CBF3, also termed dehydration-responsive
531 element-binding factors, have been well-documented in plants. In *Arabidopsis*, CBF2
532 represents a negative regulator for LT response, while CBF1 and CBF3 are positive
533 regulators (Novillo et al., 2004; Novillo et al., 2012). Interestingly, increased
534 expression of *CBF1* and *CBF3* and repressed expression of *CBF2* were observed in
535 the *BnTR1* transgenic lines (**Figure 4E-G**), indicating that BnTR1 represented a
536 unique influence on CBF members. Both alleviated accumulation of ROS and
537 activated SOD enzyme system was observed in the *BnTR1* transgenic lines (**Figure 6**),
538 suggesting active impacts of BnTR1 on the ROS scavenging system. In addition,
539 ectopic expressing *BnTR1* also promote the expression of the genes associated with
540 plant photosynthesis (**Figure 5**). Specifically, the decrease of *RCA* transcripts leads to
541 lower A_n value, which in turn slows down plant growth (Von Caemmerer et al., 2005;
542 Yin et al., 2010). Moreover, *RCA* enhances growth and photosynthesis under
543 moderate heat stress conditions (Kumar et al., 2009; Kurek et al., 2007). However,
544 overexpression of *SBPASE* improves sugar accumulation and enhanced
545 photosynthesis efficiency (De Porcellinis et al., 2018; Feng et al., 2007; Miyagawa et
546 al., 2001). In the present study, PSII was severely repressed during freezing treatment,
547 whereas the *BnTR1* transgenic *Arabidopsis* plants still exhibited higher Fv/Fm level
548 compared to WT (**Figure 5A**). These observations suggest that BnTR1 triggered a
549 series of responses including the ROS scavenging system, CBF pathway and
550 photosynthetic processes. However, further work is required to confirm their roles in
551 LT tolerance.

552

553 **Protective role of alkaloids under LT conditions**

554 Previous studies have been instrumental in revealing some metabolites
555 underlying stress response mechanisms (Thalman and Santelia, 2017). So far,
556 definitions of alkaloids are generally focused on strong pharmacological effects, such
557 as antimutagenic, antidote, anticancer and antioxidants (Schlager and Drager, 2016). The
558 concentration of alkaloid compounds is predominantly inducible when plants are
559 subjected to multiple stresses (Cheng et al., 2018; Srivastava and Srivastava, 2010).
560 However, few studies have recognized the positive correlations between alkaloids and
561 stress resistance. Application of sanguinarine for *Arabidopsis* seedlings under heat
562 stress condition could markedly enhance the tolerance, which presumably by
563 promoting the expression of heat shock proteins like HSP70 and HSP90.1S (Hara and
564 Kurita, 2014; Matsuoka et al., 2016). BnTR1 is predicted to encode a tropinone
565 reductase, which is involved in the metabolic pathway of atropine alkaloids. The
566 current study determined the total alkaloids content under stress conditions, which
567 showed an increased level in *BnTR1* transgenic lines under normal and freezing stress
568 conditions compared with WT plants (**Figure 7A**). Moreover, the application of
569 exogenous atropine alleviated the damage caused by extreme temperature in both
570 *Arabidopsis* and rapeseed seedlings (**Figure 7B, D**), which was in agreement with the
571 observations in sanguinarine under heat stress conditions (Schlager and Drager, 2016).
572 However, more studies are still required to confirm that alkaloids could function as a
573 protectant for plants to confer stronger resistance to LT stresses. The current study has
574 compared the expression level of stress-related genes in *Arabidopsis* plants treated
575 with exogenous atropine under freezing conditions (**Supplementary Figure S6**). It
576 was found that atropine could promote the expression of *CBF1*, *CBF3*, *CAB1*, *CAB3*,
577 *CAB4*, *SPASE* before or after freezing treatment, but the extent is much lower than
578 that induced by BnTR1 (**Figure 4, Figure 5**). Thus the results confirmed at least in
579 part the protective role of atropine for a plant in adaptation to LT stress. It is proposed
580 that BnTR1 works as an effector via metabolizing alkaloids accumulation,
581 photosynthesis, CBF/DREB pathways and ROS scavenging system in stressed
582 *Arabidopsis*, which in turn contributes to the adaptation under LT conditions.

583

584 CONCLUSIONS

585 During overwintering for the semi-winter type rapeseed grown in China, the
586 extremely low temperature has a deleterious impact on plant productivity.
587 Therefore, the identification of genes responsible for stress response is the prime
588 interest of researchers. Despite of limited phenotypic data, our associative
589 transcriptomics approach has been successfully used to dissect the genetics of
590 photosynthetic-related traits under low temperature conditions. The first short-chain
591 dehydrogenase/reductase, BnTR1 was identified in rapeseed, which improved the
592 transpiration rate and freezing tolerance of *Arabidopsis* plants. Taken together, our
593 findings illustrated the molecular mechanism of plant adaption to low temperature
594 stress. Finally, this work sheds light on the way to increase low temperature tolerance
595 in rapeseed by genetic engineering strategies.

596

597 **Supplementary data**

598 Supplementary Figure S1. Temperature record during the phenotypic investigation of
599 the field-grown association panel.

600 Supplementary Figure S2. Phenotype investigation of six rapeseed accessions under
601 LT stress conditions.

602 Supplementary Figure S3. Expression analysis of candidate genes in six rapeseed
603 accessions under LT stress conditions.

604 Supplementary Figure S4. Sequence alignment of BnTR1 and SDR proteins.

605 Supplementary Figure S5. Spatio-temporal expression patterns of *BnTR1* in various
606 tissues of rapeseed.

607 Supplementary Figure S6. Expression analysis of LT- or photosynthetic-related genes
608 in *Arabidopsis* with atropine application.

609 Supplementary Table S1. Information of 123 accessions used in the association study.

610 Supplementary Table S2. Summary of experiment design for the present study

611 Supplementary Table S3. Primers used in the present study.

612 Supplementary Table S4. Phenotypic variations of photosynthesis-related traits in 123

613 rapeseed accessions.
614 Supplementary Table S5. Correlation analysis of photosynthesis-related traits in 123
615 rapeseed accessions.
616 Supplementary Table S6. SNP markers detected by association study on
617 photosynthesis-related traits in rapeseed.
618 Supplementary Table S7. GEMs markers detected by association study on
619 photosynthesis-related traits in rapeseed.
620 Supplementary Table S8. Candidate genes detected by association study on
621 photosynthesis-related traits in rapeseed.

622

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629

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965

In review

966 **FIGURE LEGENDS**

967 **Figure 1.** Phenotypic variation of photosynthetic gas exchange parameters in 123
968 rapeseed accessions. Trait definition: Net photosynthesis rate (A_n), Stomatal
969 conductance to water vapour (G_{sw}), Internal CO₂ concentration (C_i), Transpiration rate
970 (E).

971

972 **Figure 2.** Manhattan plots for AT analysis in 123 rapeseed accessions. Manhattan
973 plots from left to right, represented for A_n , G_{sw} , C_i and E using SNPs (upper section)
974 and GEMs (bottom section), respectively. The $-\log_{10}$ (P -values) were plotted against
975 the position of the SNPs or GEMs on 19 chromosomes of *Brassica napus*. The black
976 line represents the $-\log_{10}$ (P -values) converted Bonferroni significance threshold for
977 SNP (5.41) and GEM (4.73), respectively.

978

979 **Figure 3.** Expression and allelic variation of *BnTR1* in rapeseed. (A) Expression
980 analysis of *BnTR1* (homolog of *Cab026133.1*) in six accessions corresponding to Hap
981 1 and Hap 2 under freezing conditions. The name of accessions 1-6 was Sv706118,
982 Kajsa, Callypso, Libritta, Gefion and Jupiter, respectively. *ACTIN* gene was used as
983 an internal control. Bars indicate the SE of three biological replicates. Different letters
984 indicate significant differences at $P < 0.05$ (one-way ANOVA with Tukey's multiple
985 comparisons test). (B) Correlation analysis between Transpiration rate (E) value and
986 expression level of *Cab026133.1* in the association panel ($n=123$). R^2 indicates the
987 coefficient of determination in linear regression. (C-D) Allelic variations at *BnTR1*
988 formed two main haplotypes and their effects on E value.

989

990 **Figure 4.** *BnTR1* confers freezing tolerance in *Arabidopsis*. (A) Expression analysis
991 of the *BnTR1* transgenic plants (L1, L3, L5) and WT plants under normal condition
992 (i.e. 23°C). (B) Investigation of transpiration rate (E) value in the *BnTR1* transgenic
993 lines and WT plants under normal conditions. (C) Performance of the transgenic lines
994 and WT plants before and after freezing treatment (-4°C for 4 h). Scale=2 cm. (D)
995 Survival rates of the transgenic lines and WT plants after freezing treatment. (E-I)

996 Relative expression levels of *CBF1* (E), *CBF2* (F), *CBF3* (G), *COR15* (H), *RD29A* (I)
997 in the transgenic lines and WT plants before and after freezing stress with the
998 *Arabidopsis ACTIN* gene used as an internal control. Normal represents 23°C,
999 freezing treatment represents 4 h at -4°C, recovery represents 3 d of recovery at 23°C.
1000 Bars indicate the SE of three biological replicates. Significant differences are
1001 determined by Student's *t*-test (**P* < 0.05, or ***P* < 0.01).

1002

1003 **Figure 5.** Variation of photosynthetic related traits and genes expression pattern in
1004 *BnTR1* transgenic plants. (A) Fv/Fm ratio in the transgenic lines and WT plants under
1005 freezing stress conditions. (B-H) Relative expression levels of *BnTR1* (B), *RCA* (C),
1006 *SBPASE* (D), *CAB1* (E), *CAB2* (F), *CAB3* (G), *CAB4* (H) in the transgenic lines and
1007 WT plants before and after freezing stress treatment with *Arabidopsis ACTIN* gene
1008 used as an internal control. L1, L3, L5 represent three independent homozygous lines
1009 of *BnTR1* transgenic plants. Bars indicate the SE of three biological replicates.
1010 Significant differences are determined by Student's *t*-test (**P* < 0.05, or ***P* < 0.01).

1011

1012 **Figure 6.** Physiological characterization of *BnTR1* transgenic plants under freezing
1013 stress conditions. (A-F) Investigation of the proline content (A), soluble sugar content
1014 (B), relative leakage (C), DAB staining analysis (D), H₂O₂ content (E), SOD activity
1015 (F) in the transgenic lines and WT plants under freezing stress conditions. L1, L3, L5
1016 represent three independent homozygous lines of *BnTR1* transgenic *Arabidopsis*
1017 plants. Bars indicate the SE of three biological replicates. Significant differences are
1018 determined by the Student's *t*-test (**P* < 0.05, or ***P* < 0.01).

1019

1020 **Figure 7.** BnTR1 mediates alkaloid accumulation and exogenous atropine application
1021 enhances freezing tolerance. (A) Total alkaloids accumulation in *BnTR1* transgenic
1022 lines and WT plants under freezing stress conditions. L1, L3, L5 represent three
1023 independent homozygous lines of *BnTR1* transgenic *Arabidopsis* plants. (B)
1024 Phenotypes of *Arabidopsis* WT plants with exogenous atropine application (0 nmol
1025 per plant, 10 nmol per plant, 30 nmol per plant) under freezing stress conditions.

1026 Scale=2 cm. (C) Survival rates of *Arabidopsis* WT plants with exogenous atropine
1027 application after the freezing treatment. (D) Phenotypes of rapeseed WT plants with
1028 exogenous atropine application (0 nmol per plant, 50 nmol per plant, 150 nmol per
1029 plant) under LT conditions. Scale=5 cm. Bars indicate the SE of three biological
1030 replicates. Significant differences are determined by Student's *t*-test (* $P < 0.05$, or
1031 ** $P < 0.01$).

In review

Figure 1.JPEG

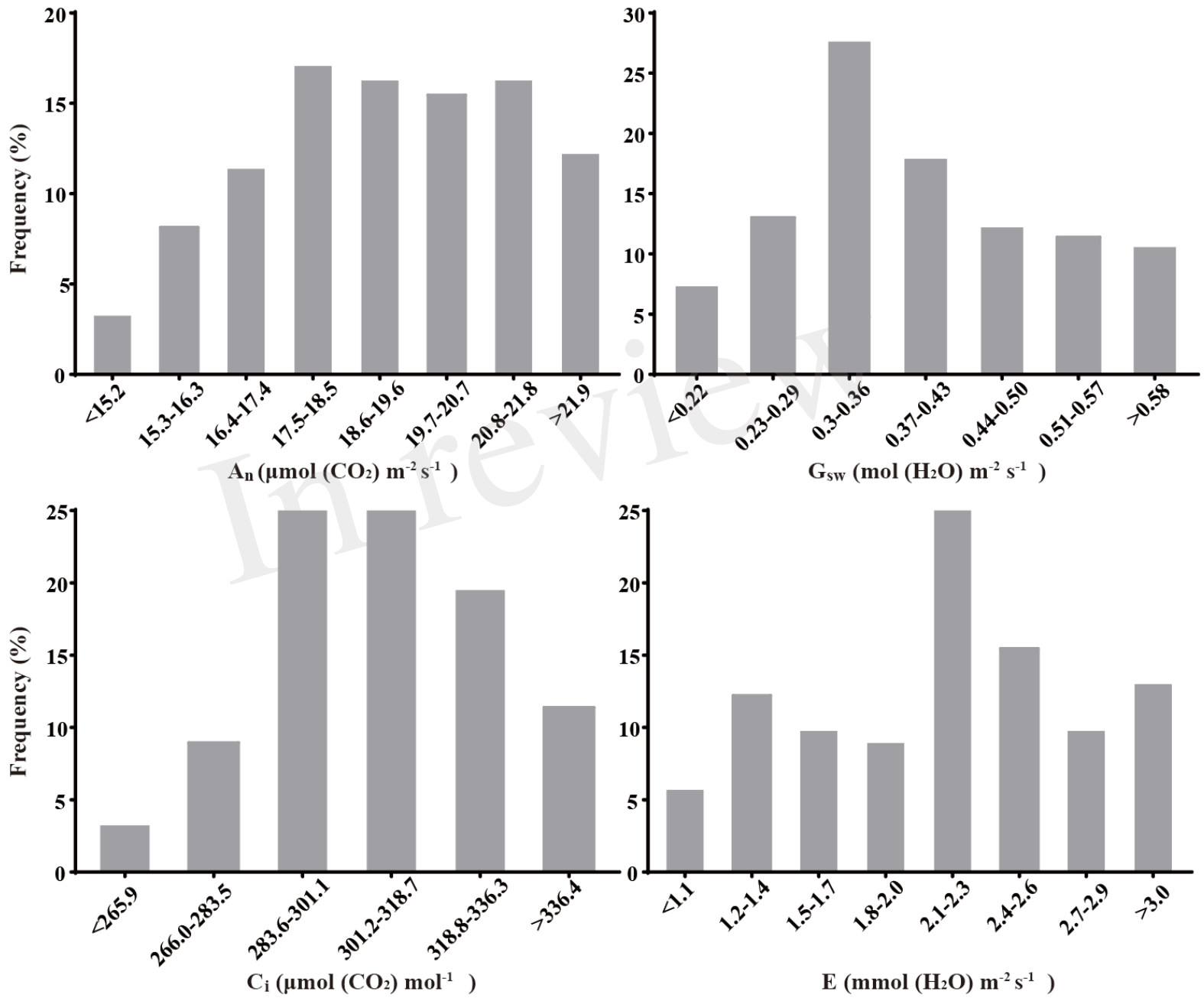


Figure 2.JPEG

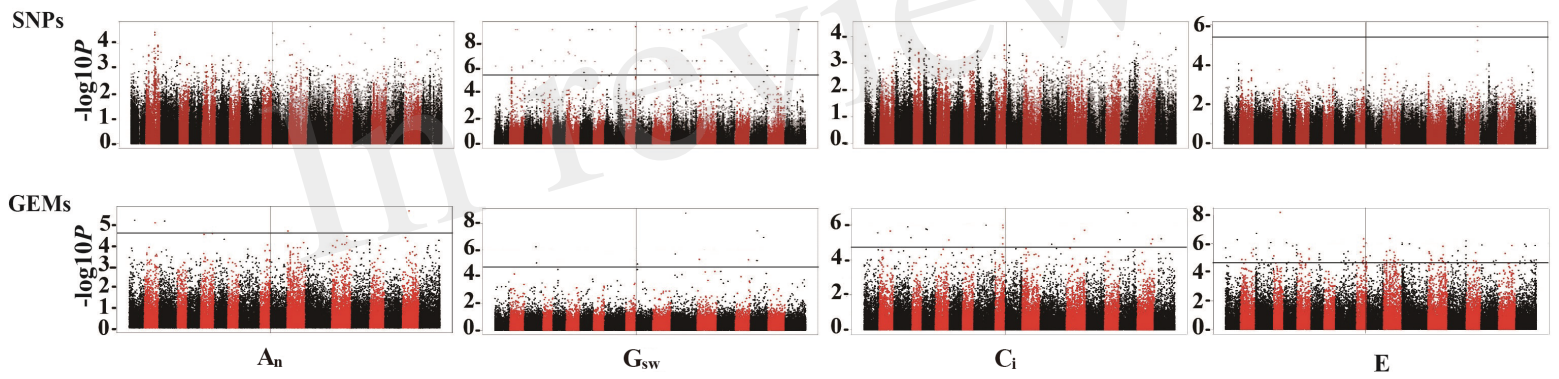


Figure 3.JPEG

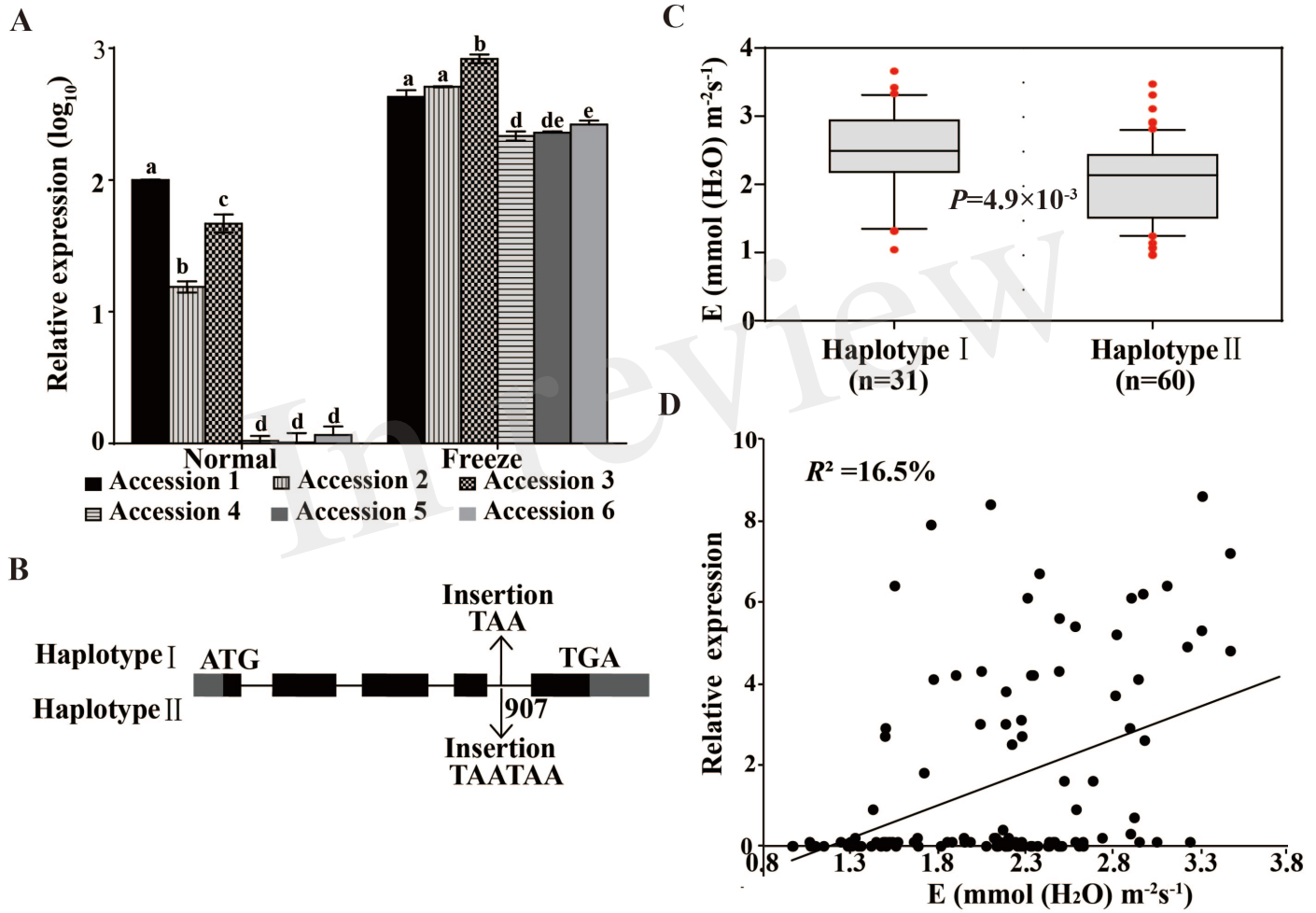


Figure 4.JPEG

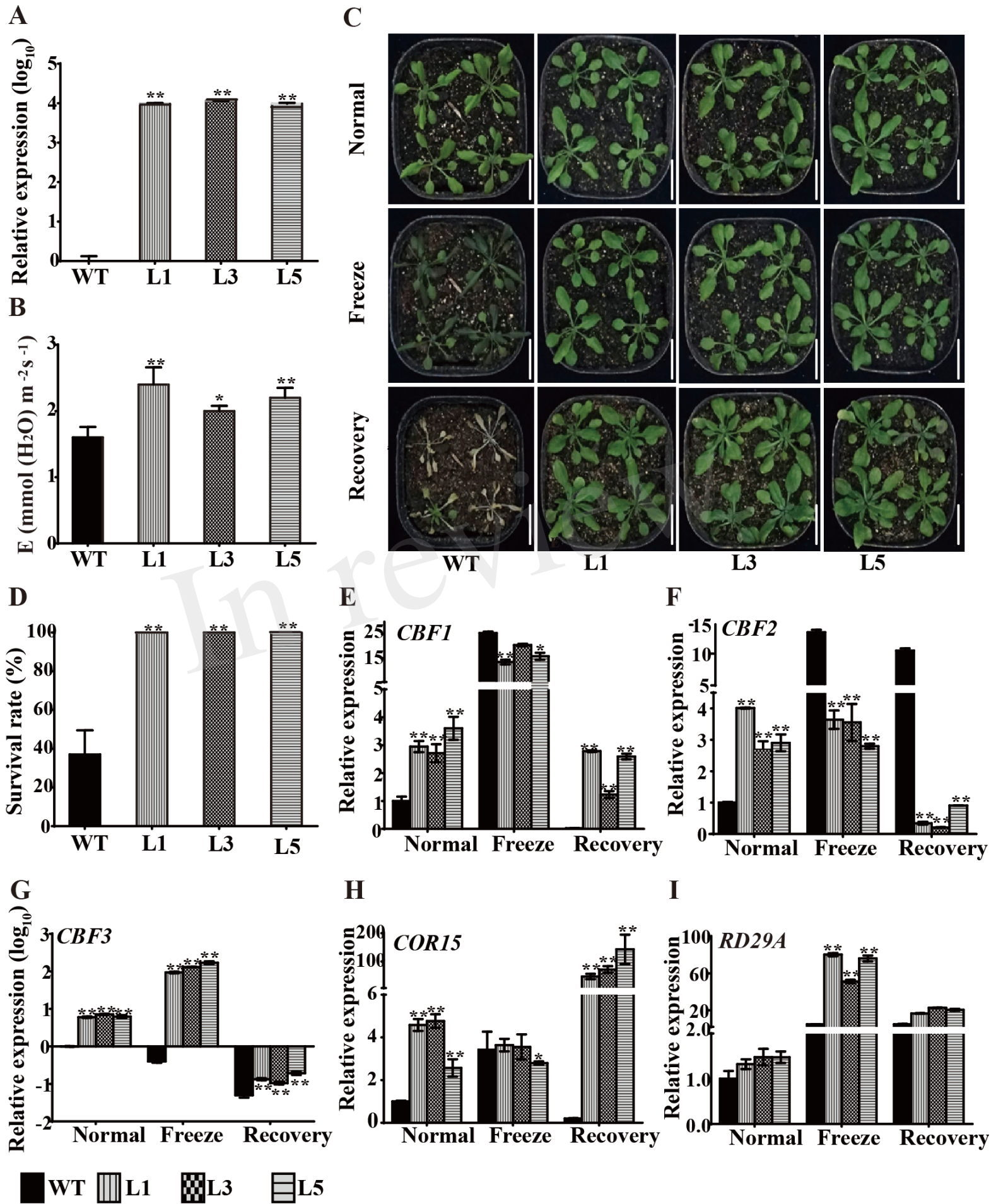


Figure 5.JPEG

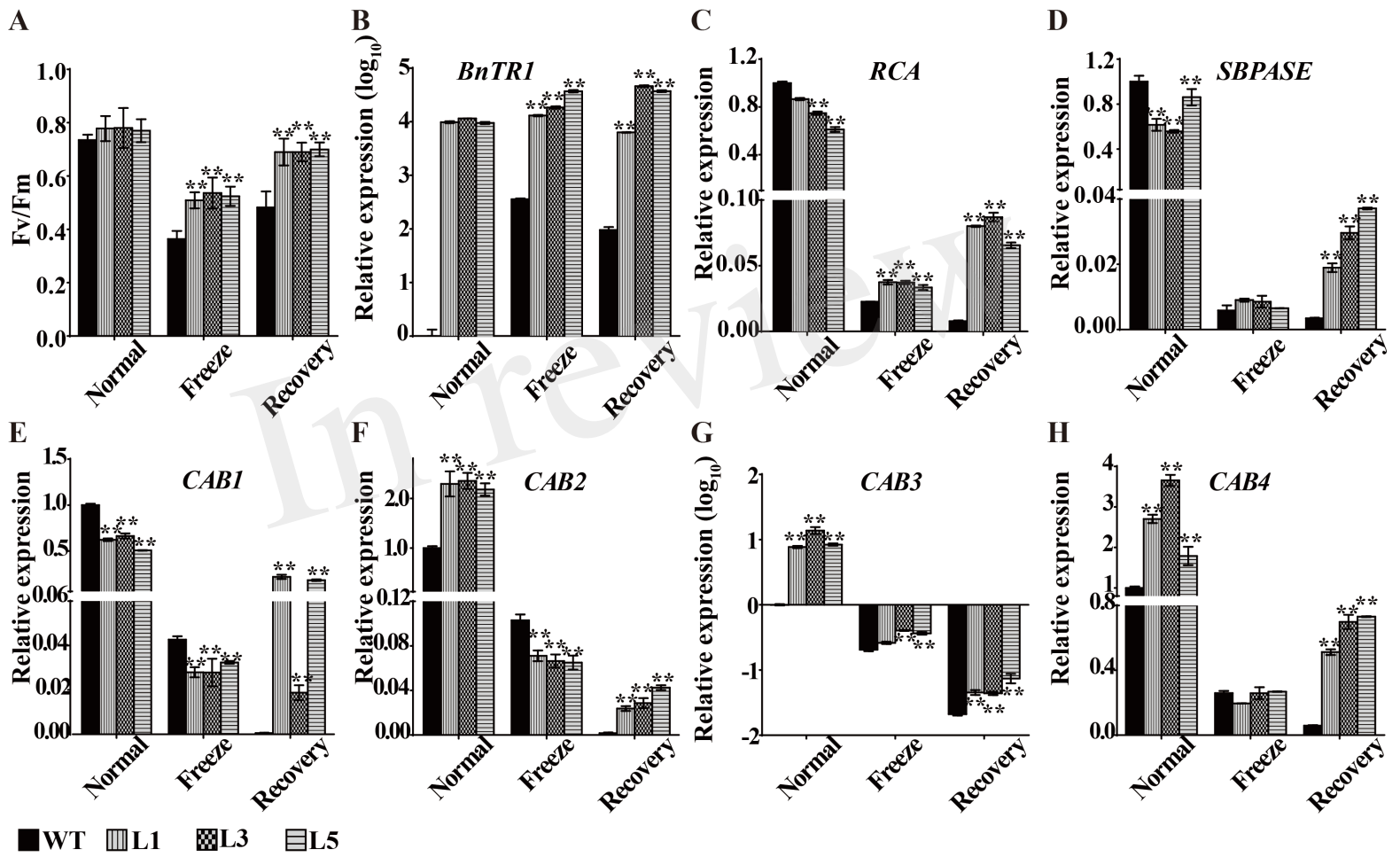


Figure 6.JPEG

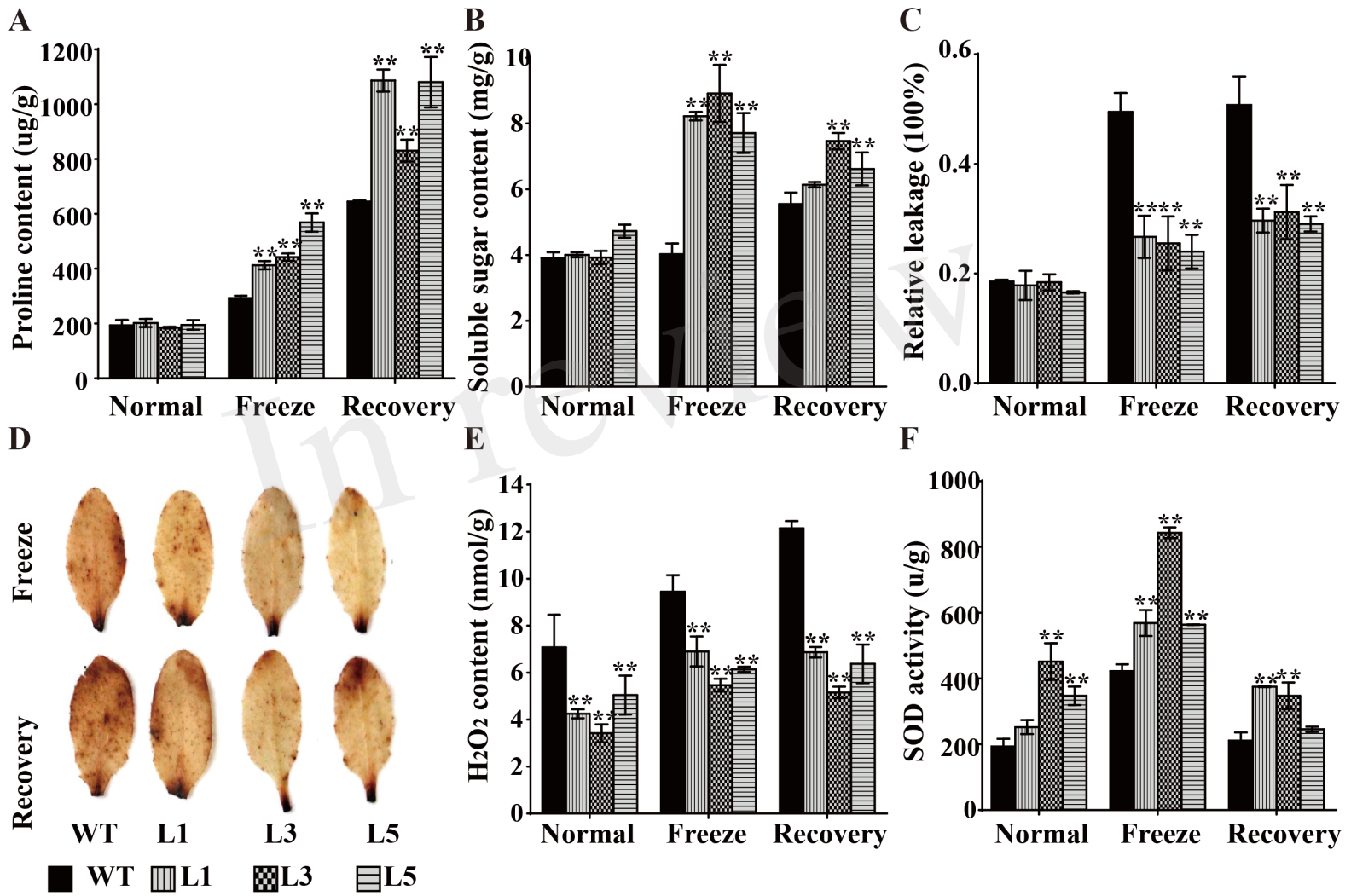


Figure 7.JPEG

