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2 **BREVIPEDICELLUS and ERECTA mediate expression of**
3 **AtPRX17 in preventing Arabidopsis callus retardation and**
4 **browning**

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24 **Running title:** *BREVIPEDICELLUS* and *ERECTA* controlling expression of
25 *AtPRX17*

Highlight: *BREVIPEDICELLUS* and *ERECTA* are involved in regulating Arabidopsis callus browning by controlling expression of *AtPRX17*.

ABSTRACT

Efficient *in vitro* callus generation is fundamental to tissue culture propagation, a process required for plant regeneration and transgenic breeding for desired phenotypes. Identifying genes and regulatory elements that prevent callus retardation and browning is essential to facilitate the development of *in vitro* callus systems. Here we show that *BREVIPEDICELLUS* (*BP*) and *ERECTA* (*ER*) pathways in *Arabidopsis* callus are converged to prevent callus browning and positively regulate an isoperoxidase gene *AtPRX17* expression in the rapid growth callus. Loss of functions in both *BP* and *ER* resulted in markedly increasing callus browning. Transgenic lines with *pro35S::AtPRX17* in the *bp-5 er105* double mutant background fully rescued this phenotypic abnormality. Using plant *in vitro* DNA-binding assays, we observed that BP protein bound directly to the upstream sequence of *AtPRX17* to promote its transcription during callus growth. ER is a universally presenting factor required for cell proliferation and growth, we show that *ER* positively regulates expression of a transcription factor *WRKY6*, which also directly binds to an additional site of the *AtPRX17* promoter for its high expression. Our data reveals an important molecular mechanism in regulating expression of peroxidase isozyme to reduce Arabidopsis callus browning.

Keywords: *Arabidopsis thaliana*, *BREVIPEDICELLUS*, *ERECTA*, *AtPRX17*, Peroxidase isozyme, Callus browning

52 **Introduction**

53 Callus retardation and browning are major impediments in vitro culture of plants,
 54 resulting in decreased regenerative ability, poor growth, and even death. Oxidative
 55 stress has been considered as one of major factors in induction of callus retardation
 56 and browning. During plant tissue culture, abundant ROS could accumulate in the
 57 rapid proliferation cells because of the high metabolic rate (Cosio and Dunand, 2009;
 58 Wells et al., 2010). The accumulation of ROS not only significantly slows the further
 59 cell proliferation rate, but often causes the callus browning (Laukkanen et al., 1999).
 60 However, in the natural conditions, tissues of higher plants, such as shoot and root
 61 meristems, usually experience a long-time continued and relatively fast cell division,
 62 whereas they are barely disturbed by cell browning even if the metabolic process
 63 always produces more ROS (Hirt and Apel, 2004; Wells et al., 2010; Tsukagoshi et al.,
 64 2010; Ikeuchi et al., 2013). One possibility is that plants could possess an efficient
 65 mechanism to scavenge ROS to ensure cell proliferation at a decided rate, however,
 66 very little is known about the molecular mechanism(s) for plant to eliminate the
 67 excess ROS in both meristematic tissues *in vivo* and developing callus tissues *in vitro*.
 68 The KNOTTED1-like HOMEODOMAIN (KNOX) transcription factor
 69 BREVIPEDICELLUS (KNAT1/BP) is involved in maintaining meristem of shoot
 70 apices (Smith et al., 2002), xylem fiber development (Woerlen et al., 2017;
 71 Felipo-Benavent et al., 2018; Milhinhos et al., 2019) and fruitlet abscission (Zhao et
 72 al., 2020). Overexpression of the maize KNOTTED1 gene caused a switch from
 73 determinate to indeterminate leaf cell fates (Sinha et al., 1993). In the process of
 74 meristematic growth, the continued and rapid cell proliferation occurs in some special
 75 zones, such as the peripheral zone in shoot apical meristem and the meristem zone
 76 adjacent to the root stem cells, in which BP plays a role in part by spatially regulating
 77 boundary genes (Woerlen et al., 2017). Down-regulated expression of a peroxidase
 78 encoded by *AtPRX9GF* in *bp-9* seedlings was reported (Mele et al., 2003). In addition,
 79 BP is involved in regulating expression of *SOBIR1/EVR* gene, which acts together
 80 with *ERECTA* (Terpstra et al., 2010) to regulate programmed cell death (PCD) during
 81 xylem development in Arabidopsis shoot (Milhinhos et al., 2019). Reactive oxygen

species (ROS) are emerging as intracellular signaling molecules that efficiently regulate PCD during xylem development and cell proliferation in meristem in shoots and roots (Breusegen and Dat, 2006; Tsukagoshi et al., 2009). Whether BP is involved in regulation of ROS signaling remains unknown.

Peroxidase catalyzes the reduction of H₂O₂ and protects tissues and cells from oxidative damage and plays important roles in controlling callus growth and browning (Brown *et al.*, 1993; Creissen *et al.*, 1994; Wakui *et al.*, 1999; Zhang *et al.*, 2020). However, apparently conflicting roles of peroxidase in the initiation of meristematic activity as well as with the suppression of growth on callus development were reported in previous studies (Basile, 1980; Goff 1975; Habib et al., 2014). An explanation on those inconsistent results is attributed to the occurrence of peroxidase in multimolecular forms, *i.e.* isoenzymes. Individual isoperoxidases may differ in their substrate specificity, pH optima, and distribution within cellular compartments, etc. (Kay et al., 1987). Specific isoperoxidases have been proven to be correlated with specific developmental events (Cosio and Dunand., 2009). Nevertheless, no specific isoperoxidase has been identified, isolated, and characterized with respect to its possible role in controlling callus growth and browning.

In this work, we report that a preoxidase gene *AtPRX17* plays a critical role involved in preventing callus browning. *AtPRX17* belongs to the class III peroxidase family (EC 1.11.1.7) (Tognolli et al., 2002), and we show that upregulation of *AtPRX17* is directly promoted by two types of transcription factors: one is the meristem-specific regulatory protein BP (Serikawa et al., 1996; Hay and Tsiantis, 2010), and the other is WRKY6 (Eulgem and Somssich, 2007), a transcription factor that is positively regulated by the putative receptor protein kinase ERECTA (ER).

106

107 **Materials and methods**

108 **Plant materials and culture conditions**

109 *Arabidopsis thaliana* wild-type ecotype Columbia (Col), Landsberg *erecta* (Ler) and
110 Landsberg *ERECTA* (Lan), mutant *brevipedicellus* (*bp*) *bp-1* and *bp-5* (Douglas et al.,

2002; Qi and Zheng, 2013), *er-105* (Hord et al., 2008) and transformants *proBP::GUS* (Ori et al., 2000) and *pro 35S::BP* (Lincoln et al., 1994) have been previously described. *prx17* (SALK_034684c) and *wrky6* (SALK_012997c) are (T)-DNA insertion alleles in the Columbia (Col) accession and was obtained from the Arabidopsis Biological Resource Center. 3~5 mm root segments from 7-d old *Arabidopsis thaliana* seedlings were cut and transferred to callus induction medium (CIM): MS medium (Murashige and Skoog, 1962) with 3% sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid, and 0.8% agar. Explants were incubated on CIM for 5 weeks at 25°C under dark conditions, then calli produced from the explants was subcultured on new CIM for twice. The suspension cultures for these calli were grown as described (Zhang et al., 2015). The suspension cultures were taken as stock for repeated callus formation. The cultures were then subcultured on solid medium again and callus with a diameter of about 1mm (about 7d after subculture on the new medium) was used to monitored the growth rate by weighed the fresh weight of cells.

Scanning electron microscopy

Samples were prepared as described by Wei et al. (2010). Briefly, calli that had been subcultured for 21 days was fixed in 3% glutaraldehyde-phosphate buffer saline fixative solution (pH 7.2) for overnight, then the samples were mounted on aluminum stubs and coated with gold in JEOL JFC-1600 after graded dehydration and replacement. The treated calli was observed with JEOL JSM-6360LV scanning electron microscope at 6kV.

Transmission electron microscopy

Samples were prepared as described by Bestwick et al. (1997). Briefly, calli as described above was incubated in 5 mM CeCl_3 in 50 mM 3-(N-morpholino) propanesulfonic acid (Mops) at pH7.2 for 1h. After treatment, samples were fixed in 1.25%(v/v) glutaraldehyde and 1.25%(v/v) paraformaldehyde in 50 mM sodium cacodylate (CAB) buffer, pH7.2 for 1h and post-fixed in 1%(v/v) osmium tetroxide in CAB for 45 min. After being dehydrated through an ethanol series, samples were infiltrated and embedded in Epon 812 resin. The specimens were sectioned and the

140 thick section (0.5µm) were stained with 0.1% toluidine blue and examined under light
141 microscopy (Leica DMLB). The ultra-thin sections (about 100 nm) were examined
142 using a transmission electron microscope (Hitachi 7650 TEM) at an accelerating
143 voltage of 75kV.

144 **Diaminobenzidine oxide and nitroblue tetrazolium staining**

145 In situ detection of hydrogen peroxide was performed by staining with
146 diaminobenzidine oxide (DAB) using an adaptation of a previous method (Daudi et
147 al., 2012). Briefly, calli that had subcultured for 21 days was stained for 2h in 1mg/ml
148 DAB solution containing Tween 20 (0.05% v/v) and 10 mM sodium phosphate buffer
149 (pH7.0) in a Eppendorf tube. The staining was terminated in ethanol: glycerol: acetic
150 acid 3:1:1(bleaching solution) placed in a water bath at 95°C for 15 min.

151 For nitroblue tetrazolium (NBT) staining, calli was stained for 15 min in a solution of
152 2mM NBT in 20 mM phosphate buffer pH6.1. The reaction was stopped by
153 transferring the calli in distilled water. At least six independent culture plates were
154 used as biological replicates and three callus blocks were sampled from each culture
155 plate.

156 **TUNEL assay**

157 The calli was fixed with 4%(w/v) fresh paraformaldehyde in PBS and labeled by the
158 TUNEL reaction mixture: the terminal deoxynucleotidyl transferase solution and the
159 label solution described in the kit's manual. The stained cells were analyzed with a
160 fluorescence microscope (Olympus, FV1000). The excitation and emission wave
161 lengths were 488 nm and 515 nm, respectively.

162 **Evans blue staining**

163 Cell death in calli was assayed as described by Baker and Mock (1994). Briefly, a 50
164 mg callus was added to a mixture of 0.5 ml of 1% (w/v) Sucrose and 0.5 ml of
165 0.5%(w/v) Evans blue solution. After 10 min, cells were drained and rinsed with
166 deionized water, 30-40 ml, until no further blue eluted from the cells. After washing,
167 the cells gently transferred to a 1ml plastic Eppendorf tube containing carborundum
168 (<0.1mg). 0.5 ml of 1% aqueous SDS was added to cells to release the trapped Evans
169 blue from the cells. The cells were ground and the homogenate diluted with 0.5 ml of

170 deionized water, centrifuged at 10,000×g for 3 min. A 0.8 ml aliquot of the
171 supernatant was removed and the optical density determined spectrophotometrically
172 at 600 nm.

173 **Peroxidase isoenzyme analysis**

174 The classical guaiacol peroxidase (class III peroxidases, E. C. 1.11.1.7) isoenzymes
175 were analyzed as described by Naton et al. (1992). Briefly, three-week subcultured
176 callus was homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) containing 0.58
177 mol/L sucrose. The extracts were centrifuged (10,000 g, 20 min, 4°C) and the
178 supernatant collected as the peroxidase fraction. For enzyme visualization, the
179 procedures of Brewbaker et al. (1968) were modified. Peroxidases were visualized by
180 adding 4 ml substrate solution (2% 3, 3'-diaminobenzidine tetrahydrochloride in
181 0.1mM Tris-acetate buffer, pH 4.5) to 15.2 ml dd H₂O, and starting the reaction with
182 0.8 ml 3% H₂O₂ (v/v). Incubation for 5~15 min at room temperature revealed
183 greenish-brown bands on a light-yellow background.

184 **Reverse transcription-polymerase chain reaction analyses (RT-PCR) and** 185 **qRT-PCR**

186 Total RNA was extracted from three-week subcultured callus using an RNAiso plus
187 Kit (TaKaRa, <http://www.takara.com.cn>) according to the manufacturer's instructions.
188 cDNA was synthesized using 2 µg of total RNA and 100 U of ReverTra Ace reverse
189 transcriptase (Toyobo Co., Ltd, Japan) according to the manufacturer's instructions.
190 The products were subsequently taken to amplify the targeted genes with primers for
191 *PRX17*; *PRX52*; *WRKY6*; *WRKY15*; *WRKY 25*; *WRKY 33* and *WRKY46*. The
192 constitutive housekeeping gene *ADENINE PHOSPHORIBOSYL TRANSFERASE1*
193 (*APT1*) was used as an internal control. Reactions of qRT-PCR were done in a
194 384-well plate format with 7900HT Fast Real-time PCR System (Applied
195 Biosystems®, <http://events-na.appliedbiosystems.com>), and SYBR Green to monitor
196 double-stranded DNA synthesis. The *APT1* gene was used as reference for the *BP*, *ER*,
197 *AtPRX17* and *WRKY6* genes. Experiments were conducted at least three times with
198 equivalent results. The primers used in this study are listed in Supplemental Table S1.

199 **Screening and isolation of the Arabidopsis T-DNA insertion mutants, *prx17* and** 200 ***wrky6***

201 For identification of homozygous insertion of *prx17* and *wrky6* mutants, respectively,
202 segregation analysis was performed by genotyping progeny of the mutant lines
203 containing T-DNA insertion in the *AtPRX17* gene (SALK_034684c) or in *WRKY6*
204 gene (SALK_012997) in various crosses. Progeny seedlings (about 4-week-old) were
205 genotyped by extracting DNA from a single leaf of the seedling using PCR
206 genotyping as described by Shpak et al. (2003). RT-PCR was performed to verify
207 knock-out of the *AtPRX17* and *WRKY6* transcript. The primers used in this study are
208 listed in Supplemental Table S1.

209 **35S::*PRX17* transgenic plants**

210 A complementary vector consisted of CaMV35S promoter fused to the
211 *PRX17*-encoding genomic fragment (Col-0, wild-type) was prepared according to
212 Venglat et al. (2002). Briefly, a DNA fragment containing the full-length genomic
213 coding region for *AtPRX17* gene was amplified by PCR using the Col-0 cDNA
214 template and the primers: 5'-CTGCAGATGTCTCTTCTTCCCAT-3' and
215 5'-GAGCTCTCAAGATACAAGCAATAC-3'. The amplified fragments were
216 digested with *PstI* and *SacI* and inserted into the *PstI* and *SacI* sites of
217 pMD18-T-Vector. Subsequently, the *PstI/SacI*-amplified fragment was digested by
218 *PstI/SacI* and ligated to CaMV 35S promoter of binary vector pHB. *Agrobacterium*
219 *tumefaciens* (GV3101) containing this recombinant construct was used to transform
220 *bp-5 er-105* plants as described by Clough and Bent (1998). All transgenic lines used
221 in this study are T3 homozygous plants with single copy insertion.

222 **Electrophoretic mobility shift assay (EMSA)**

223 For synthesis and purification of recombinant BP and WRKY6 proteins, cDNAs
224 containing the full-length coding region of these two proteins were amplified by PCR.
225 The amplified *BP* or *WRKY6* cDNA was cloned into the vector pET-30a (Novagen,
226 www.novagen.com) by using *BamHI* and *SacI* sites. The PCR primers for the *BP* and
227 *WRKY6* amplification were listed in Supplemental Table S1.

228 All constructs were verified by sequencing. The 6xHis-BP and 6xHis-WRKY6
229 expression plasmid was transformed into the bacterial strain BL21 (DE3) pLysS. The
230 transformed cells were cultured at 37°C until the OD₆₀₀ of the cell culture was 0.5,
231 and then induced with 1 mM IPTG for 36 h at 12°C. For extraction of native fusion
232 protein, the cultured bacteria cells were lysed by using a high-pressure cell crusher
233 and the fusion proteins were purified with Ni-NTA resin (Qiagen, www.qiagen.com)
234 according to the manufacturer's instructions.

235 For EMSA, the complementary pairs of biotin-labeled oligonucleotides corresponding
236 to the AtPRX17 promoter region containing BP or WRKY6 binding sites was obtained
237 by PCR amplification using 5'-biotin-labeled primers (Sangon Biotech, Shanghai Co.,
238 Ltd) to generate double-stranded probes. DNA binding reactions were performed with
239 the Light Shift Chemiluminescent EMSA Kit (Pierce, www.piercenet.com) according
240 to the instructions. DNA binding reactions were performed in a total volume of 20 µl
241 of buffer (10mM Tris-HCl, pH 7.5, 2.5% Glycerol, 50mM KCl, 1mM DTT, 5mM
242 MgCl₂) containing 50ng/µL poly[dI-dC], 0.05% NP-40, 1 µg of the recombinant
243 His-tagged BP or WRKY6 proteins and 20 fmol of probe. The binding specificity was
244 assessed by competition with a 100- or 200-fold excess of unlabeled double-stranded
245 oligonucleotides. Binding reaction mixtures were incubated for 30 min at 23°C and
246 separated on native PAGE gels (5% polyacrylamide gel) in 0.5×TBE buffer, at 100V
247 for 90 min. After electrophoresis, gels were bolted onto a positively charged nylon
248 membrane (Amersham, now GE Healthcare, <http://www.gelifesciences.com>). The
249 DNA was linked using a UV light cross-linker instrument equipped with 254-nm
250 bulbs for 0.8 min exposure.

251 Transient GUS assay by agroinfiltration of *Nicotiana benthamiana*

252 PRX17 promoter sequence was amplified with specific primers (the forward primer
253 5'-AAGCTT TGGGACTGAATGAACTGCTGA-3' and the reverse primer 5'-
254 GGATCCACTTTTTCTTTTTTGGTGTG-3') by PCR from Arabidopsis genomic
255 DNA and cloned into the transformation vector pCAMBIA1300-pBI101 at the
256 HindIII and BamHI restriction sites, respectively as Reporter PRX17_{pro}::GUS. The BP

257 full-length cDNA sequence was amplified with *BP*-specific primers (the forward
258 primer 5'-GAATTCATGGAAGAATACCAGCATGACAACAG-3' and the reverse
259 primer 5'-GTCGACTTATGGACCGAGACGATAAGGTCCAT-3') cloned into
260 pC1300-N1-YFP vector at EcoRI and SalI sites as Effector 35S-BP.
261 The *WRKY6* full-length cDNA sequence was amplified with *WRKY6*-specific primers
262 (the forward primer 5'-GGATCCATGGACAGAGGATGGTCTGGTCTCA-3' and
263 the reverse primer 5'-GTCGACTTGATTTTGTGTTTCCTTCGC-3') and cloned
264 into pC1300-N1-YFP vector at BamHI and SalI sites as Effector 35S_{pro}::*WRKY6*.
265 The constructs of *PRX17*_{pro}::GUS with 35S_{pro}::BP or 35S_{pro}::*WRKY6*, were
266 transformed into *Agrobacterium* (GV3101). *Agrobacteria* was infiltrated into intact
267 leaves of *Nicotiana benthamiana* as previously described (Kane et al., 2007). After
268 infiltration, plants were kept at 23°C for 3 days. Histochemical GUS assay was
269 performed as previously described (Wei et al., 2010).

270 **Chromatin Immunoprecipitation (ChIP)**

271 ChIP assay was performed using a method modified from the Chromatin
272 Immunoprecipitation (ChIP) Assay Kit (Upstate , Catalog # 17-295). Callus was
273 incubated in 1% formaldehyde for 30 min under vacuum. The cross-linking was
274 stopped by adding glycine to a final concentration of 0.125 M. Tissues were rinsed
275 with water and ground into a fine powder with liquid nitrogen. To extract chromatin,
276 the powder was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM
277 Tris, pH 8.1, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The chromatin DNA was
278 sonicated to reduce DNA length and diluted 1:10 in chromatin immunoprecipitation
279 dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl,
280 pH 8.1, 167 mM NaCl). The chromatin solution was precleared with Protein A
281 agarose beads blocked with salmon sperm DNA (Upstate Biotechnology).
282 Immunoprecipitations were performed with anti-BP antibody (sc-19215). The
283 BP-bound chromatin was purified by incubation with Protein A agarose beads
284 blocked with salmon sperm DNA and washing with low-salt wash buffer (0.1% SDS,
285 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt

286 wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1,
287 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1%
288 deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1), and TE buffer.
289 The BP-bound chromatin was eluted from Protein A agarose beads with elution buffer
290 (1% SDS, 0.1 M NaHCO₃). After reversing the cross-linking by the addition of NaCl
291 to a final concentration of 200 mM and incubation at 65°C for 4h, the DNA was
292 purified by treating with 40 mg/mL proteinase K for 1 h, following by
293 phenol/chloroform extraction and precipitation with DNA mate (TaKaRa). The
294 primers used for PCR were listed in Supplemental Table S1.

295

296 **Results**

297 **Combined action of *BP* and *ER* in controlling callus growth**

298 In the wild-type (Col), root tips as explants from 7-day-old seedlings on callus
299 induction medium (CIM) are usually able to generate the earliest callus at about 3th
300 day after culture (DAC). The callus then undergoes a rapid growth from 10 DAC to
301 24 DAC (Fig. 1A and E, Fig. S1A-F), accompanied by rapidly increased *BP*
302 expression in new regeneration callus (Fig. S1G-J). The first appearance of calli from
303 the same-age root explants of *bp-5* and *er-105* mutants as well as *bp-5 er-105* double
304 mutant (Col background) was also at about 4 DAC and the callus grew normally as
305 wild-type (Fig. S2). However, callus of double mutant *bp5 er105* showed significantly
306 retarded growth rate and browning appearance after 3-4 times subculture (Fig. 1D),
307 while *bp-5* and *er-105* callus grew normally as their wild-type (Fig. 1A, B and C) .
308 Compared with those of Col, *bp-5*, and *er-105*, the total fresh weight produced by the
309 *bp-5 er-105* explants was significantly reduced (Fig. 1E). In addition, we also
310 analyzed another loss-of-function mutant of *BP* (*bp-1*), which is in the Landsberg
311 *erecta* (Ler) genetic background, and similar to the *bp-5 er-105* double mutant, callus
312 growth of *bp-1* exhibited a markedly slowed manner in comparison with calli of
313 *bp/ER* mutant, *Ler (BP/er)* and *Lan (BP/ER)* (Fig. S3). These results indicated that *BP*
314 and *ER* could co-regulate the growth and browning of Arabidopsis callus.

Hydrogen peroxide and superoxide in relation to BP/ER controlling callus growth

The histological examination indicated that the cell arrangement of *bp-5 er-105* calli was smaller and denser in the outer layer, but flaccid in the central region (Fig. 1J) compared to its wild-type Col (Fig. 1F). Observations using scanning electron microscopy (SEM) revealed that Col calli consisted of many globular nodules (Fig. 1G), whereas *bp-5 er-105* calli had fewer globular nodules (Fig. 1K). Several previous studies indicated the role of ROS homeostasis in the callus development (Tang et al., 2004; Che et al., 2006; Zhang et al., 2018). To test whether the distribution of H₂O₂ in *bp-5 er-105* callus cells was different from that in wild-type, we used the cerium chloride assay, in which cerium chloride forms electron-dense precipitates in the presence of H₂O₂ through formation of cerium perhydroxide (Bestwick et al., 1997; Shen et al., 2015). With this assay, we observed apparently localized cerium precipitations in the *bp-5 er-105* callus cells (Fig 1O and P), especially in the cell wall (Fig 1P), in comparison with that of wild-type Col callus cells (Fig. 1H and I). This result indicated highly accumulation of H₂O₂ in cells of *bp-5 er-105* double mutant during rapid growth. High level of H₂O₂ could induce programmed cell death (PCD), which can be judged based on DNA fragmentation using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Biswas and Mano, 2015). More than 50% of the cells of 21 DAC *bp-5 er-105* callus had positive TUNEL staining, while less than 20% of the cells of wild-type (Col) and two single mutants *bp-5* and *er-105* callus displayed positive TUNEL staining (Fig. 2A). The ratio of death cells in *bp-5 er-105* callus was apparently higher than those of Col, *bp-5* and *er-105* callus, as detected by Evans blue (Fig 2B). To understand whether the defective callus growth and cell death of *bp-5 er-105* is related to the accumulation of H₂O₂ in the cells, we analyzed H₂O₂ and superoxide (O₂⁻) levels in *bp-5 er-105* callus by comparing with those in Col, *bp-5* and *er-105* callus, respectively. H₂O₂ level in *bp-5 er-105* callus was apparently higher than those in Col, *bp-5* and *er-105* callus (Fig. 2D), whereas the O₂⁻ level in *bp-5 er-105* callus was lower compared with those in Col, *bp-5* and *er-105* (Fig. 2C).

345 These results indicated that the function of H₂O₂ scavenge in *bp-5 er-105* double
346 mutant may be defective during rapid growth stage of callus.

347 ***BP* and *ER* promote expression of *AtPRX17* during callus growth**

348 The class III peroxidases (E. C. 1.11.1.7, PRXs) in plant tissue play important roles
349 in removal of excess H₂O₂ for maintaining a balance of ROS in tissue culture and the
350 activities of peroxidase isoenzymes are important factors related to callus growth
351 (Kay and Basile, 1987; Tournaire et al., 1996; Che et al., 2006).

352 We examine the patterns and activities of peroxidase isoenzymes in Col, *bp-5* and
353 *er-105* and *bp-5 er-105* by a native polyacrylamide gel electrophoresis (PAGE)
354 followed by in-gel 3, 3'-diaminobenzidine tetrahydrochloride (DAB) staining. Several
355 bands corresponding to DAB-oxidizing active proteins were observed, among which
356 one isoenzyme band was apparently weaker in *bp-5 er-105* callus than those in Col,
357 and the corresponding single mutants *bp-5* and *er-105* callus (Fig. 3). These
358 observations suggested that a specific peroxidase isoenzyme might be affected in *bp-5*
359 *er-105* during callus growth.

360 To know if expression of some *PRX* genes in *bp-5 er-105* callus was altered at the
361 transcription level, we re-examined expression levels of peroxidase genes in
362 Arabidopsis callus according to microarray data published by previous studies (Che *et*
363 *al.*, 2002; Che *et al.*, 2006). Among of the listed 73 genes of Arabidopsis class III
364 PRXs, *peroxidase17* (*AtPRX17*, At2g22420) and *peroxidase52* (*AtPRX52*,
365 At5g05340) were obviously up-regulated from 4 to 10 DAC during callus growth (Fig.
366 S4), with the stage equivalent to that of rapid callus growth in our study (Fig. 1E).

367 Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that
368 expression of *AtPRX52* in *bp-5 er-105* callus was not different from that in Col, *bp-5*
369 and *er-105* callus (Fig. 4A), while expression of *AtPRX17* was apparently
370 down-regulated in the callus of *bp-5 er-105*, compared with that of Col, *bp-5* and
371 *er-105* (Fig. 4A and B). *AtPRX17* was selected for further study. A Salk T-DNA line
372 (designated as *prx17*, SALK_034684c) was identified as homozygous by PCR
373 analysis and there was no expression of *AtPRX17* in the mutant callus (Fig S5). The
374 growth rate of *prx17* callus was also apparently reduced in comparison with wild-type

Col (Fig. 4E) and similar to that of *bp-5 er-105* callus (Fig. 1E). The patterns of PAGE showed that the dramatically reduced intensity of a band in *prx17* mutant callus was consistent with that in *bp-5 er-105* callus (Fig 4C). Thus, it is possible that the peroxidase isoenzyme AtPRX17 (isoPRX17) is defective in *bp-5 er-105* callus. To test the possibility of AtPRX17 expression regulated by *BP* and *ER*, we constructed a fusion with the AtPRX17 cDNA under the control of the 35S promoter (*35S_{pro}::PRX17*), and introduced this fusion into the *bp-5 er-105* double mutant. A total of 12 independent transgenic plants were obtained, from which three homozygous lines for *35S_{pro}::AtPRX17* were subsequently identified for further characterization (Fig. 5A). The activities of peroxidases isoenzymes in cell cultures of these three transgenic lines were further examined, and the putative isoPRX17 band was rescued (Fig. 5B). Additionally, the callus growth and cell death defects in the *35S pro:PRX17/bp-5 er-105* transgenic plants were all rescued compared with those in Col and *bp-5 er-105* double mutant plants (Fig. 6A and B). These results indicated that *BP* and *ER* maintain normal callus growth possibly via promoting expression of AtPRX17.

391

392 **BP binds to the TGAC motif in the AtPRX17 promoter**

393 To investigate whether *BP* directly regulates AtPRX17 expression, we
394 computationally searched for the nucleotide sequence similar to that of the published
395 BP-binding *cis*-element from the genebank (www.arabidopsis.org) in AtPRX17
396 promoter (Smith et al., 2002). Three motifs consisting of TGACANCT (N=A or T)
397 were identified in AtPRX17 promoter region between -1732 bp and -1359 bp from
398 the translation initiation site, including TGACAACCT from -1712 to -1704,
399 TGACAACT from -1680 to -1673, and TGACATCT from -1392 to -1385 (Fig. 7A).
400 The interactions *in vitro* between the BP and the AtPRX 17 promoter region were
401 further examined by electrophoretic mobility shift assays (EMSAs), using the
402 recombinant BP proteins. The DNA fragment of the AtPRX17 promoter region from
403 -1732 to -1359 bp, which contains three BP-binding motifs, was used as probe.

As shown in Figure 7A, a retarded band in the presence of the recombinant BP protein was observed. However, in the presence of excess amounts of the homologous unlabeled DNA fragment as competitor, the bound probe in the retarded band was apparently competed away. To further examine the function of BP in regulation of *AtPRX17* expression *in vivo*, a transient expressional test was performed based on *agro*-infiltration of *Nicotiana benthamiana* leaves as described by the previous methods (Voinnet et al., 2003; Kane et al., 2007). In particular, tobacco leaves were co-infiltrated with two constructs: the reporter construct *AtPRX17_{pro}::GUS* and the effector construct *35S_{pro}::BP* (Fig. 7B). After infiltration and a 2-day recovery, the infiltrated leaves were collected for GUS staining, and GUS activity could reflect the *AtPRX17* promoter activity. GUS staining revealed that reporter construct alone only resulted in relatively low *AtPRX17* expression (Fig. 7C, left panel), whereas GUS staining apparently increased in tobacco leaves when co-infiltrated with both effector and reporter constructs (Fig. 7C, right panel). These results further supported that BP could directly regulate *AtPRX17* gene expression. To provide more evidences that BP binds to the *AtPRX17* promoter, we performed a chromatin immunoprecipitation (ChIP) assay using wild-type callus, and our results confirmed that BP could bind to *AtPRX17* promoter *in vivo* (Fig. 7D).

The ER-downstream target *WRKY6* also directly regulates the *AtPRX17* expression

ER protein is a membrane-bound leu-rich repeat receptor-like Ser/Thr kinase known as a pleiotropic regulator of multiple developmental and physiological processes and as a modulator to respond to environmental stimuli (Torii et al., 1996; Nanda et al., 2019). Previous studies show that ER regulates a set of *WRKY* transcription factor genes including *WRKY6*, *15*, *25*, *33*, and *46* (Terpstra et al., 2010). To test whether ER modulates callus growth through *WRKY* genes, we first analyzed the expression of *WRKY* genes. Compared with Col, expression of *WRKY6* was apparently down-regulated in *er-105* and *bp-5 er-105* callus (Fig. 8A), whereas expression of several other *WRKY* genes was also analyzed, such as, *WRKY15*, *WRKY25*, *WRKY33*

434 and *WRKY46*, but their expression was not significantly affected (Fig. 8A; Fig S6).
 435 Quantitative real-time PCR analysis demonstrated that expression of *WRKY6* in Col
 436 callus was gradually increased during 3 ~9 DAC, and attained to the highest level at 9
 437 DAC, coincident with expression pattern of *ER* during callus growth (Fig. 8B,
 438 compared to Fig. 1E). Thus, *WRKY6* was selected for further study.
 439 A mutant line with loss of *WRKY6* function, here referred to *wrky6*, was obtained (Fig.
 440 S7A and B), and used to construct *bp-5 wrky6* double mutant. Expression of *WRKY6*
 441 was severely reduced in *wrky6* and *bp-5 wrky6* double mutant (Fig S7C). The activity
 442 of the band corresponding to *AtPRX17* in the *bp-5 wrky6* callus was apparently
 443 reduced, similar to that in *bp-5 er-105*, but markedly lower than those of the Col, *bp-5*,
 444 and *wrky6* (Fig. 8C). Furthermore, the growth rate of the *bp-5 wrky6* callus was
 445 significantly decreased compared with that of wild-type callus, but similar to that of
 446 the *bp-5 er-105* callus (Fig S8). These results indicated that *WRKY6* is involved in
 447 regulating the expression of *AtPRX17*.
 448 To further explore whether *WRKY6* also directly regulates the *AtPRX17* gene, we again
 449 examined the *AtPRX17* promoter, trying to identify nucleotide sequence that *WRKY6*
 450 binds to. Two previously reported WRKY-binding cis-motifs, TTGACC (Robatzek *et*
 451 *al*, 2002), were found in the *AtPRX17* promoter between -1119 to -1114 and between
 452 -1005 and -1000 from the translation initiation site (Fig. 9A). The physical
 453 interaction between the *WRKY6* and *AtPRX17* promoter region was examined by
 454 EMSA as we have described above for the BP protein. The DNA fragment of the
 455 *AtPRX17* promoter region from -1141 to -981 was used as probe. As shown in Figure
 456 8A, a retarded band in the presence of the recombinant *WRKY6* protein was observed.
 457 In the presence of excess amounts of the unlabeled competitor fragment, the amount
 458 of the labeled retarded complexes was obviously reduced (Fig. 9A). These results
 459 suggested that *WRKY6* also specifically binds to the *AtPRX17* promoter.
 460 To test the possible *WRKY6* function in directly regulating *AtPRX17* expression *in*
 461 *vivo*, we performed the transient assay through agro-infiltration of *Nicotiana*
 462 *benthamiana* leaves with the *35S_{pro}::WRKY6* effector and *PRX17_{pro}::GUS* reporter
 463 constructs (Fig. 9B). GUS staining revealed that reporter construct alone only resulted

in relatively low *AtPRX17* expression (Fig. 9C, left panel), whereas GUS staining apparently increased in tobacco leaves when co-infiltrated with both effector and reporter constructs (Fig. 9C, right panel). Our results showed that similar to the *35S_{pro}::BP* effector, *35S_{pro}::WRKY6* effector also enhanced the expression of *AtPRX17*.

Discussion

The class III peroxidases (PRXs) are a kind of plant-specific oxidoreductase that is involved in a broad range of physiological processes throughout the plant life cycle, including lignification, suberization, auxin catabolism, wound healing and defense against pathogen (Kay and Basile, 1987; Hiraga et al., 2001; Tognolli et al., 2002; Welinder et al., 2002; Passardi et al., 2006; Almagro et al., 2009; Cosio and Dunand, 2010; Herrero et al., 2013). Recent advances indicated that the processes directly or indirectly targeted by PRXs include gene expression, post-transcriptional reactions, and switching or tuning of metabolic pathways and other cell activities (Liebthal et al., 2018). However, little is known about the signal transduction for regulating expression of PRX genes. Previous studies show that the promoter region of peroxidase genes contains the transcription factor (TF) binding sequence. For example, AGL2 and /or WUS binding sites were detected in the promoter region of *PRX13*, *PRX30* and *PRX55* (Cosio and Dunand, 2010), but the specific function and precise role of these TFs remains unclear. In this study, we provide new evidences that BP together with WRKY6 directly regulate the expression of *AtPRX17* in Arabidopsis callus by binding to its promoter region.

Callus is like the meristematic tissues, which is a self-renewing structure consisting of stem cells and their immediate daughters (Springer and Kohn, 1979; Xiao et al., 2020), but the mechanism on how to maintain this self-renewing structure is unclear.

Reactive oxygen species (ROS) play an important role in maintaining plant cell proliferation (Vemoux et al., 2000; Wells et al., 2010). In Arabidopsis, two main ROS, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide exhibit distinct patterns of distribution in root tissues (Dunand et al., 2007). $O_2^{\cdot-}$ and H_2O_2 mainly accumulate in dividing and expanding cells in the meristem and elongation zones of root tips, respectively (Wells

et al., 2010). Peroxidases have been suggested to be related with production (Mäder et al., 1980; Bolwell et al., 1995) and scavenging (Mehlhorn et al., 1996; Kvaratskhelia et al., 1997) of hydrogen peroxide and play an important role in the cell proliferation and senescence of higher plants (Abeles et al., 1988; Oh et al., 1997; Kay and Basile, 1987; Tsukagoshi et al., 2010). The class III peroxides multigene family in Arabidopsis genome show specific expression patterns in different developmental stages and organs (Tognolli et al., 2002). For example, in callus from Arabidopsis root explant, expression of *PRX73*, *PRX ATP21a*, *PRX27*, *PRXATP8a*, *PRX13a*, *PRXATP11a* and *ATP19a* were obviously down-regulated, while expression of *PRXATP12a* was up-regulated (Che et al., 2006). *PRX1*, which was mediated by *ABI3*, has been found to be specifically expressed in the embryo and aleurone layer during maturation and desiccation stage of development (Haslekås et al., 2003). Although these previous studies have found the appearance or disappearance of specific peroxidase isoforms during a particular process or in a particular localization (Loukii et al., 1999; Allison & Schultz, 2004), it is difficult to associate the band observed on an PAGE gel with a particular protein and define their roles in certain specific developmental processes, because protein purification of peroxidase isoenzymes is not straight forward as well as no obvious quantitative relationship exists between the transcript expression level and the protein activity (Dunand et al., 2003; Cosio and Dunand, 2009). In this study, either mutation of *AtPRX17* gene in *prx17* mutant or impaired isoAtPRX17 in double mutants *bp-5 er-105* or *bp-5 wrky6* resulted in an apparently decrease in the growth rate of callus (Fig 1E, Fig S8). Concentration of H₂O₂ in the isoPRX17 deficiency mutant callus was apparently higher than that in Col (Fig 1P, Fig 2C and D). Thus, we assumed that isoPRX17 could be essential in maintaining ROS homeostasis during Arabidopsis callus development. We further found that *AtPRX17* could be specific in *BP/ER* pathway. *BP* has previously been shown to be redundant with *ER* to influence inflorescence stem and pedicel differentiation through local regulation of the level, or response to, a vasculature-associated growth inhibitory signal (Douglas et al., 2002; Douglas and Riggs, 2005). Recently *BP* and *ER* were found to involve in preventing precocious

initiation of fiber differentiation during wood development (Vera-Sirera et al., 2019). However, which signaling pathway is involved in BP/ER pathway is still not clear. In this study, *BP* and *ER* are functionally redundant in promoting callus growth and inhibiting tissue browning. Analysis of the peroxidase isoenzyme patterns by a native PAGE gel indicated that double mutations in *BP* and *ER* genes resulted in deficiency of a PRX isoenzyme band, which is corresponding to AtPRX17 isoenzyme (iso PRX17). Furthermore, the absence of isoPRX17 band in *bp-5 er-105* double mutant callus could be rescued in the *35S_{pro}::PRX17/bp-5, er-105* transgenic lines. Thus, *BP* and *ER* could regulate expression of *AtPRX17* gene by affecting its promoter activity. Although the BP recognition site is unknown, three binding sites for the KNOX gene *BP/KNAT1* (TGACAG(G/C)T) (Smith et al., 2002; Bolduc et al., 2012) are present at location -1732 bp to -1359 bp relative to the putative transcription start site in the promoter of *AtPRX17*, suggesting that BP might directly bind to the *AtPRX17* promoter region. Further EMSAs and transient assays using tobacco leaves also demonstrated that BP could directly bind to the *AtPRX17* promoter region and affect its activity. However, the down-regulation of *AtPRX17* transcript level was only observed in *bp-5 er-105* double mutant, but not in either *bp-5* or *er-105* single mutant. This implied that *AtPRX17* could be a target of both BP and ER proteins. ER protein is a membrane-bound leu-rich repeat receptor-like Ser/Thr kinase (LRR-RLK; Torii et al., 1996; Van Zanten et al., 2009), but not a transcript factor (TF), it is impossible to regulate the expression of *AtPRX17* gene by directly binding to the promoter region. TFs regulated by ER have been reported (Terpstra et al., 2010). For example, *WRKY6* was suggested to act downstream of *ER* (Terpstra et al., 2010). In our study, two *WRKY6* binding sites (TTGACC) (Robatzek et al., 2002) close to one *KNAT1* binding site (TGACATCT) were also observed in the promoter of *AtPRX17* from -1141bp to -981bp upstream of the putative transcription start site. In addition, *WRKY6* protein bonded to the DNA segment of *AtPRX17* promoter was also found with a much higher affinity *in vitro* and *vivo*. These observations suggest that the function of ER convergently with BP to influence callus growth could be via *WRKY6*, which directly regulate expression of *AtPRX17* gene.

Previous studies indicate that KNOX TFs in plant have degenerate binding sites and acquire specificity through cooperation with binding partners, as found in animals (Bolduc et al., 2009; Moens and Selleri, 2006). For example, KNOX proteins bind DNA as heterodimers with BELL proteins, another class of TALE HD protein (Bellaoui et al., 2001; Smith et al., 2002). KNOX and BELL share similar in vitro consensus binding sites, and their heterodimerization increases their affinity for DNA (Smith *et al.*, 2002; Viola & Gonzalez, 2006). In this study, mutation of *BP* could cause inhibition of callus proliferation and *AtPRX17* band deficiency, but this was only observed in an *er-105* or *wrky6* background. WRKY6 likely acts downstream of ER and as a cofactor of BP in regulating expression of *AtPRX17* gene during callus development. Further studies should clarify the mechanism of how ER regulates WRKY6 in Arabidopsis callus, and whether BP interacts with WRKY6 to form complex for regulating *AtPRX17* in vivo.

567

Supplementary data

Fig. S1. Callus was induced from Arabidopsis root explants.

Fig. S2. Callus induction from root explants of wild-type Col, single mutants *bp5* and *er-105* and double mutant *bp5/er-105* after 21 days on the callus induce medium.

Fig. S3. Growth of a double mutant *bp-1* and their single mutant *Ler (BP/er)* and *bp/ER*, respectively, in comparison with their wild-type (Lan)..

Fig. S4. Time-resolved cluster analysis showing ratios of callus-developing related increases of Class III peroxidases in transcript abundances.

Fig. S5. Identification of homozygous insertion mutants at the PRX17 locus.

Fig. S6. RT-PCR analysis of WRKY25, WRKY33 and WRKY46 expression in the callus of *er-105* and Col, respectively.

Fig. S7. Identification of homozygous insertion mutants at the WRKY6 locus

Fig. S8. Comparison of growth rate of *bp-5 wrky6* double mutant callus with that of *bp-5 er-105* and *prx17* mutant callus, respectively.

Table S1. Oligonucleotide primers used in this study.

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590

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592 investigated gene expression; LW, YW and CM participated in investigation, HZ
593 conceived of the study, participated in its design and coordination, and drafted the
594 manuscript. All authors have read and agreed to the published version of the
595 manuscript.

596

597 **Conflicts of Interest:** The authors declare that they have no conflict of interest.

598

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599 **Figure Legends**

600 **Fig.1** *BP* and *ER* genes implicate in callus growth and browning. A-D Callus of
601 wild-type Col (A), single mutant *er-105*(B) and *bp5* (C) and double mutant *bp5 er-105*
602 (D) after 3 weeks cultured on MS medium. Note that double mutant *bp5 er-105* callus
603 appears smaller and oxidative browning (D). Scale bars, 10 mm. E The growth rate of
604 culture cells among the double mutant (*bp-5 er-105*) , single mutant (*er-105* and *bp-5*)
605 and Col. Error bars indicate the Standard deviation of the mean for three
606 replications($n \geq 20$ calli for each time point). (F and J) Resin sections of Col (F) and
607 *bp5 er-105* (J) callus. Scale bars, 100 μ m. G and K Scanning electron microscopy
608 images of Col (G) and *bp5er-105* (K) callus. Scale bars, 100 μ m.

609
610 **Fig. 2** Comparison of cell death and endogenous O_2^- and H_2O_2 content in callus of
611 *bp-5 er-105* with Col, *bp-5* and *er-105*. (A) Typical TUNEL assay fluorescence microscopy
612 images of the 21-day calli and their phase-contrast microscopy (DIC) images. Scale bars,
613 200 μ m. (B) Evaluation of cell death in 21-day calli of Col, single mutants (*er-105* and *bp-5*)
614 and double mutant (*bp-5 er-105*). The callus was stained with Evans blue (EB) as described in
615 Methods. Cellular uptake of Evans blue was quantified by spectrophotometry at OD₆₀₀ nm.
616 Values represent means $\pm$ standard deviation (SD) (n=10; student's t-test **p < 0.001). (C

617 and D) Quantification of nitroblue tetrazolium (NBT) and diaminobenzidine oxide (DAB
618 staining intensity of Col, bp5, er105 and bp5 er105, respectively. The staining intensity of Col
619 is given as 100%, of which the staining intensity of mutants was compared, respectively.

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621

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623 Col. (A) Native PAGE gels stained for peroxidase activity. Note that a band (asterisk indicating) in
624 double mutant *bp-5 er-105* callus was weaker significantly in comparison with that of *bp-5*, *er-105*
625 and Col callus, respectively.

626 (B) Protein stained with Coomassie Brilliant Blue (CBB) was used as loading control.

627

628 **Fig. 4** The absence of a peroxidase isoenzyme band of *bp-5 er-105* double mutant calli is
629 consistent with that of *prx17* mutant calli. (A) The expression analysis of *PRX17* and *PRX52*
630 genes in callus of different genotypes by RT-PCR. Note that expression of *PRX17* gene was
631 apparently down-regulated in *bp-5 er-105* double mutant in comparison with that in *er-105*, *bp-5*
632 and Col callus, respectively. Total RNA samples were extracted from callus grown on a new
633 medium for 14 days after subcultured. (B) qRT-PCR analysis of *PRX17* transcription levels in
634 *bp-5er-105* in comparison with that in *er-105*, *bp-5* and Col calli, respectively. Values represent
635 means $\pm$ standard deviation (SD) (n=3) and results were consistent in at least three biological
636 replicates. (C) Isoenzyme analysis of peroxidases in *bp-5 er-105*, *er-105*, *bp-5*, Col and *prx17*
637 mutant callus by PAGE (50 μ g proteins/lane). Note that a band (arrows point) is absent in both
638 *bp-5 er-105* and *prx17* in comparison with that in *er-105*, *bp-5* and Col, respectively.

639 (D) A represent band in SDS-PAGE visualized by staining with Coomassie brilliant blue (CBB)
640 was used as loading control. (E) Growth rate of *prx17* culture cells in comparison with that of Col.
641 Error bars indicate the Standard deviation of the mean for three replications (n=16 calli for each
642 time point; *p < 0.05, **p < 0.001, student's t test).

643

644 **Fig. 5** Substitution of *PRX17* promoter with 35S promoter in *bp-5 er-105* double mutant could
645 rescue the loss band of the peroxidase isoenzyme in the native PAEG gel. (A) RT-PCR analysis of

646 *PRX17* expression in *35S::PRX17/bp-5 er-105* transgenic lines(*bp-5 er-105* background). Total
647 RNA samples were isolated from 14-day -old subculture callus. **(B)** Analysis of peroxidase
648 isoenzymes in callus of *35S::PRX17/bp-5,er-105* transgenic line and the indicated mutants by
649 PAGE (50µg proteins/lane). Noted that the lost band (asterisks indicate) of peroxidase
650 isoemzymes in *bp-5 er-105* and *prx17* callus was rescued in the *35S::PRX17/bp-5 er-105*
651 transgenic lines (e.g. line #8, #9 and #10) (arrow indicates). A represent band in SDS-PAGE
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653

654 **Fig. 6** The growth rate of *prx17* mutant callus and *35S::PRX17* callus in comparison with that of
655 *bp-5 er105* double mutant callus and wild-type Col callus, respectively.

656 **(A)** Comparison of growth rate (expressed as an increase in callus fresh weight) of callus cultures
657 derived from *bp5 er-105*, Col, *prx17*, *35S::PRX17* root explants. 0.1g fresh weight callus of each
658 indicating genotype was inoculated on the new CIM medium for 8 days and then measured the fresh
659 weight. The increase in fresh weight of Col is given as 1.0, of which other mutants were compared,
660 respectively. Values represent means $\pm$ standard deviation (SD) (n=15~20).

661 **(B)** Evaluation of cell death by Evans blue (EB) staining. Callus inoculated with new medium for
662 2 weeks was stained with Evans blue as described in Methods. Cellular uptake of Evans blue was
663 quantified by spectrophotometry. Values represent means $\pm$ standard deviation (SD) (n=10 calli) of
664 three experiments.

665

666 **Fig. 7** Interaction of KNAT1 protein with the promoter region of *PRX17*. **(A)** Electrophoretic
667 mobility shift assay (EMSA) for KNAT1 binding to the *PRX17* gene promoter. The probe,
668 biotin-labeled DNA corresponding to the *PRX17* gene promoter region (–1732 to –1359) from the
669 site of initiation of translation and with three potential KNAT1 binding sites (box1, box2 and
670 box3), was incubated in the absence (lane 1) and in the presence (lanes 2-4) of recombinant
671 full-length KNAT1 proteins (His-KNAT1). As competitor DNA, homologous 100- and 200-fold
672 excess of unlabeled DNA fragment was added to the reaction mixtures, respectively. The black
673 arrows indicate shift band. The white arrows indicate free probe (FP). **(B)** Effector and Reporter
674 constructs used in the transient assays. *pro35S*, Promoter from the *35S* gene of cauliflower mosaic
675 virus; GUS, β -glucuronidase. **(C)** Effect of KNAT1 on *PRX17* promoter activity in vivo.

676 *Nicotiana benthamiana* intact leaves were infiltrated with *Agrobacterium* strains carrying the
677 reporter construct with or without effector constructs. *proPRX17::GUS*, without effector;
678 *proPRX17::GUS+pro35S::KNAT1 CDS*, with effector. Transactivation activity was detected by
679 GUS staining assay. **(D)** Chromatin Immunoprecipitation (ChIP) assay of the Col callus showed
680 that KNAT1 bound to *PRX17* promoter *in vivo* by qRT-PCR. Immunoprecipitation was performed
681 with anti-KNAT1 antibody (Anti-KNAT1) or without antibody (no Ab). Primers (F+R) used in
682 ChIP assay as shown in (A). Values represent means $\pm$ standard deviation (SD) (n= 3).

683

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685 Arabidopsis callus. **(A)** RT-PCR analysis of *WRKY6* and *WRKY15* expression in the *er-105* and Col,
686 respectively. *APT1* was used for the control. Total RNA samples from 14-day subculture old callus
687 were isolated. **(B)** Relative transcript abundance changes of *WRKY6* gene in developing callus were
688 detected using qRT-PCR. Callus were generated from root explants, and then inoculated on the new
689 medium for 3, 6, 9 and 12 days. Values represent means $\pm$ standard deviation (SD) (n= 3). **(C)**
690 Analysis of peroxidase isoenzymes in callus of indicated samples by PAGE (50 μ g proteins/lane).
691 Noted that the lost band (asterisks indicate) of PRX17 isoenzyme in *bp-5 wrky6*, like that in *bp-5*
692 *er-105* callus (arrow indicates).

693

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695 mobility shift assay (EMSA) for WRKY6 binding to the *PRX17* gene promoter. The probe,
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697 site of initiation of translation and two WRKY6 binding sites (box 4 and box 5), was incubated in
698 the absence (lane 1) and in the presence (lanes 2-4) of recombinant full-length WRKY6 proteins.
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704 infiltrated with *Agrobacterium* strains carrying the reporter construct with or without effector

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709

710

Figure 1

Fig.1 BP and ER genes implicate in callus growth and browning.

(A-D) Callus of wild-type Col (A), single mutant *er-105* (B) and *bp-5* (C) and double mutant *bp5 er-105* (D) after 3 weeks cultured on MS medium. Note that double mutant *bp5 er-105* callus appears smaller and oxidative browning (D). Scale bars, 10 mm.

(E) The growth rate of culture cells among the double mutant (*bp-5 er-105*), single mutant (*er-105* and *bp-5*) and Col. Error bars indicate the Standard deviation of the mean for three replications ($n \geq 20$ calli for each time point).

(F and J) Resin sections of Col (F) and *bp5 er-105* (J) callus. Scale bars, 100 μm .

(G and K) Scanning electron microscopy images of Col (G) and *bp5er-105* (K) callus. Scale bars, 100 μm .

(H and O) Electron micrographs of sections of Col (H) and *bp5 er-105* (O) callus, which was stained by 10 mM cerium chloride (CeCl_3) to compare the localized H_2O_2 in cells between Col (H) and *bp5 er-105* (O) calli. Twenty calli from each genotype was collected for analysis of resin sections and electron microscopy. Scale bars, 20 μm . (I) and (P) are enlarged from (H) and (O), respectively. Arrows point that the deposits are formed throughout cell wall of *bp5 er-105* callus cells, but less in cell wall of Col callus cells. Scale bars, 2 μm .

Figure 2

Fig. 2 Comparison of cell death and endogenous O_2^- and H_2O_2 content in callus of *bp-5 er-105* with *Col*, *bp-5* and *er-105*.

(A) Typical TUNEL assay fluorescence microscopy images of the 21-day calli and their phase-contrast microscopy (DIC) images. Scale bars, 200 μ m.

(B) Evaluation of cell death in 21-day calli of *Col*, single mutants (*er-105* and *bp-5*) and double mutant (*bp-5 er-105*). The callus was stained with Evans blue (EB) as described in Methods. Cellular uptake of Evans blue was quantified by spectrophotometry at OD₆₀₀ nm. Values represent means $\pm$ standard deviation (SD) (n=10; student's t-test **p < 0.001).

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Figure 3

Fig. 3 Isoenzyme analysis of peroxidases in developing callus of *bp-5 er-105*, *bp-5*, *er-105* and Col.

(A) Native PAGE gels stained for peroxidase activity. Note that a band (asterisk indicating) in double mutant *bp-5 er-105* callus was weaker significantly in comparison with that of *bp-5*, *er-105* and Col callus, respectively.

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