1 ***StPOPA*, encoding an anionic peroxidase in *Solanum tuberosum*,** 2 **enhances resistance against *Phytophthora infestans***

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9 **Abstract**

10 Potato late blight, caused by *Phytophthora infestans*, is one of the major threats
11 affecting the quality and output of potato all over the world. Reactive oxygen species
12 (ROS) acted as a signal molecule to transmit signals in plants at the early stage of
13 disease infection, and it could induce disease resistance of the plant, including potato
14 late blight. Anionic peroxidases in many plants were reported to be involved in
15 defense to disease. However, limited information about anionic peroxidase genes is
16 available for the potato. Here, we reported that the expression of the *StPOPA*, a gene
17 encoding a suberization-associated anionic peroxidase, was associated with resistance
18 in potato against *P. infestans*. The *StPOPA* gene was induced by *P. infestans* infection,
19 mechanical damage, jasmonic acid and ethylene treatment. Overexpression of the
20 *StPOPA* gene in potato enhanced the resistance against *P. infestans* via promoting the
21 accumulation of callose in the cell wall and ROS in the cytoplasm, which restricted
22 the infection and spreading of the disease possibly by purposeful programmed cell

death. Taken together, our results suggested that the *StPOPA* gene contributed to potato immunity against *P. infestans* and this gene could be used for the genetic improvement of resistance against potato late blight.

Introduction

Potato (*Solanum tuberosum*) is the fourth largest food crop in the world. However, late blight, caused by the oomycete pathogen *Phytophthora infestans*, is seriously affecting potato production all over the world (Jiang et al., 2018). Unfortunately, no effective ways to control late blight have yet been found except for frequent use of chemicals. The most efficient strategy to prevent the disease is to develop crop varieties with durable and broad-spectrum resistance (Dangl et al., 2013; Fukuoka et al., 2009).

Plants are attacked by bacteria, viruses, fungi, oomycetes and other pathogenic microorganisms in their lives and could defend themselves via multiple resistance mechanisms (Giraldo and Valent, 2013). Despite the lack of a cellular immune system, plants share with animals an innate immune system (Li et al., 2017). There are two major innate immune responses in plants: the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Boller and He, 2009) and the effector-triggered immunity (ETI) (Jones and Dangl, 2006). The PTI and ETI phases of plant immunity may be spatiotemporally distinct, but they are both intimately related to the reactive oxygen species (ROS) burst (Grant and Loake, 2000; Kadota et al., 2014; Nürnberger et al., 2004; Torres et al., 2006).

As one part of the plant defense response mechanism, the outbreak of reactive

oxygen species is closely related to the occurrence of the anaphylactic reaction and programmed cell death (Lamb and Dixon, 1997) and production of ROS in plant cells marks the successful recognition of plant pathogens and activation of plant defenses (Torres, 2010). Doke first demonstrated pathogen-induced apoplastic ROS production in potato tuber tissues (Doke, 1983) and indeed, ROS was reported to act as signal transduction molecules in plant defense response (Frederickson Matika and Loake, 2013; Lehmann et al., 2015; Mittler et al., 2011; Torres, 2010).

Peroxidases (EC1.11.1.7) can catalyze the redox reaction of hydrogen peroxide with various inorganic hydrogen or organic hydrogen donors (Jouili et al., 2011). Welinder divided peroxidases superfamily into three categories: Class I, Class II and Class III. Class I peroxidases exist in the mitochondria, chloroplasts and bacteria. Class II peroxidases exist in fungi while Class III peroxidases are typical plant peroxidases (Welinder, 1992). Burel divided peroxidases into three types according to the isoelectric point: neutral ($pI=7$), alkaline or cationic ($pI>7$) and acid or anionic ($pI<7$) (Burel et al., 1994).

Peroxidases, as the important component of plant cells, have many physiological functions (Pandey et al., 2017; Passardi et al., 2005). They play an important role in the process of active oxygen metabolism (Inupakutika et al., 2016). Peroxidase can catalyze the NADH or NADPH in the cell wall to generate molecular oxygen and reactive oxygen species through a series of reactions (Montillet and Nicole, 2000). A large number of studies have showed that the activity of peroxidase is positively related to plant disease resistance (Almagro et al., 2009; Jwa and Hwang, 2017;

67 Oliveira et al., 2017). The anionic peroxidase gene in tomato was reported to be
68 induced by exogenous pathogenic fungi elicitor and H₂O₂ for the first time (Mohan
69 and Kolattukudy, 1990). Expression of a tobacco anionic peroxidase gene was
70 reported to be tissue-specific and developmentally regulated (Klotz et al., 1998). A
71 highly anionic peroxidase was cloned in potato, with induced expression in suberizing
72 potato tubers and tomato fruits. It was suggested to be involved in the deposition of
73 the aromatic domain of suberin (Roberts et al., 1988). A few years later, the
74 biochemical characterization of this suberization-associated anionic peroxidase of
75 potato was reported (Bernards et al., 1999). However, few anionic peroxidase genes in
76 potato were reported to be involved in late blight resistance except that
77 RNA-silencing of anionic peroxidase gene M21334 was showed to decrease the
78 potato plant resistance to *P. infestans* (Sorokan et al., 2014). More evidences of
79 anionic peroxidase involved in potato disease resistance remain to be defined.

80 In our previous cDNA-AFLP study, a putative suberization-associated anionic
81 peroxidase precursor (POPA) gene was found uniquely up-regulated by *P. infestans*
82 (Li et al., 2009), which was further demonstrated by virus-induced gene silencing
83 (VIGS) with TRV in *Nicotiana benthamiana* and potato to decrease the resistance to
84 late blight (Du et al., 2013). Here we reported the cloning of this newly identified
85 anionic peroxidase gene named *StPOPA* from potato and evidenced that the *StPOPA*
86 gene enhanced potato resistance against *P. infestans* via promoting the accumulation
87 of callose in cell wall and ROS in cytoplasm.

88 **Materials and methods**

89 **Plant materials, pathogenic bacteria and plant treatments.** *S. tuberosum* cultivar
 90 E-potato 3 (E3) was used in this study and transgenic E3 of 35S:GUS was used as
 91 empty vector control. Two physiological races of *P. infestans*, Ljx18 (1.3.4.7) and
 92 HB09-14-2 (1.2.3.4.5.6.7.8.9.10.11), collected from Hubei Province, China, were
 93 used to infect the transgenic and control plants.

94 To generate overexpression plants of the *StPOPA* gene, full-length cDNA was
 95 cloned using the primers (sequences of all the primers used in this study were listed in
 96 Supplementary Table S1) designed according to the PGSC sequence
 97 PGSC0003DMC400039701 from E3 and ligated to vector PBI121 via the restriction
 98 endonuclease site of *Bam*HI and *Sac*I. To generate RNA-interference plants of the
 99 *StPOPA* gene, 170-bp non-conservative region of it and 308-bp conservative region of
 100 both the *StPOPA* gene and its homologous gene *StTAP2* were cloned with the primers
 101 and separately ligated to vector pHELLSGATE12 via recombination reaction. All the
 102 vectors mentioned above were transformed into *Agrobacterium tumefaciens*
 103 LBA4404.

104 For the biotic and abiotic stress treatments, leaflets with similar size on the third
 105 fully expanded leaves below the apical apex were excised carefully from the potato
 106 plants and sampled at 0, 6, 12, 24, 36, 48, 60 and 72 hours after infected by *P.*
 107 *infestans* and at 0, 3, 6, 9, 12, 24, 36, 48 and 60 hours after treated by SA (10 mM),
 108 ETH (100 µM), ABA (100 µM) and JA (50 µM). Sampled leaves were immediately
 109 frozen in liquid nitrogen and stored in -70 °C refrigerator till use.

110 ***Agrobacterium*-mediated genetic transformation of potato**

111 *A. tumefaciens* LBA4404 containing target vectors (pBI121-*StPOPA*
112 pHELLSGATE12-*StPOPA* and pHELLSGATE12-*StPOPA-TAP2*) were used to infect
113 potato via microtuber discs transformation method (Tian et al., 2015). At first these
114 agrobacterium strains were inoculated into 2 mL YEB liquid medium with 50 mg/L
115 kanamycin (Kan) and 50 mg/L rifampicin (Rif), and the medium was cultured at
116 28 °C with 200 rpm rotation rate for 20 hours. Then 0.5 mL bacterial fluid was
117 transferred into 50 mL fresh YEB liquid medium with 50 mg/L Kan, and the medium
118 was cultured at 28 °C with 200 rpm rotation rate until the OD 600 reached 0.5.

119 *A. tumefaciens* bacterial fluid was centrifuged at 6,000 g at 4 °C for 6 minutes.
120 Mycelium was then resuspended in equal volume of MS liquid medium (containing
121 3% sucrose). Seven to nine-week-old test-tube potatoes were cut into thin discs of 1-2
122 mm thick and these potato discs were soaked in *A. tumefaciens* bacterial fluid for 10
123 minutes. Positive lines were first screened on selective medium (MS + 3% sucrose + 1
124 mg/L indole-3-acetic acid (IAA) + 0.2 mg/L gibberellic acid (GA3) + 0.5 mg/L
125 6-benzylaminopurine (6-BA) + 2 mg/L zeatin (ZT) + 75 mg/L Kan + 400 mg/L
126 cefotaxime (Cef, PH 5.9) and then transferred to rooting medium (MS + 3% sucrose
127 + 50 mg/L Kan + 200 mg/L Cef, PH 5.9), were then confirmed by the PCR with the
128 primers of 35S promoter (the primers are shown in the Supplementary Table 1).

129 ***P. infestans* infection assays**

130 *P. infestans* strain Ljx18 and HB09-14-2 (14-2) were cultured on rye and sucrose agar
131 (RSA) medium at 18 °C for 13 days before collecting the inoculum for potato
132 infection as previously described (He et al., 2015). Sporangia were collected from

133 medium and washed with ddH₂O to a concentration of 7×10^4 per mL and then 0.01
134 mL sporangia suspension was used to inoculate potato leaves. Vernier caliper was
135 used to measure the lesion length (L) and width (W). Lesion area (LA) was calculated
136 by the formula $LA = 0.25 \times \pi \times L \times W$. All the data were analyzed by ANOVA in the
137 software SPSS.

138 **DNA, RNA extraction and real-time RT-PCR**

139 Genomic DNA was extracted from potato leaves by the CTAB method and total RNA
140 was extracted from potato leaves by the TRI pure Reagent (Aidlab). The first strand of
141 cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen) according
142 to the manufacturer's instructions. Quantitative PCR was conducted on a CFX
143 connectTM Real-Time PCR Detection System (Bio-Rad) using SYBR Premix ExTaq
144 (TaKaRa) according to the manufacturer's instructions. The potato *EF-1α* gene was
145 used as the internal control (primers were listed in Supplementary Table S1). The
146 relative expression level was calculated as reported previously (Livak and Schmittgen,
147 2001).

148 **Transient expression in *Nicotiana benthamiana* and subcellular localization**

149 Full-length CDS of the *StPOPA* gene was amplified with the adapters of attB1 and
150 attB2, and then the target fragment was reorganized to target vector pK7FWG2, with
151 GFP tag fusion expressed. Sequence confirmed vector was transformed into *A.*
152 *tumefaciens* GV3101 and the single clone was cultured in 4 mL medium at 28°C with
153 200 rpm rotation rate. After 12 hours, bacterial fluid was harvested and resuspended
154 in appropriate volume of MMA solution (10 mM MgCl₂, 10 mM MES (Morpholine

155 sulfonic acid), 200 μ M AS (acetosyringone), pH 5.6) to adjust the OD600 of bacterial
156 fluid to 0.01. Then, mix the target bacterial fluid with the p19 (gene silencing
157 suppressor) (Voinnet et al., 2010) bacterial liquid with the volume ratio of 1:2. After 2
158 hours at room temperature, mixed bacterial liquid was injected into epidermis of
159 *Nicotiana benthamiana*. After 36 hours of static growth, epidermis was torn off to be
160 observed by fluorescence confocal microscope LSM510 (Carl Zeiss AG), GFP
161 excitation light wavelength was 488 nm.

162 **Histochemical staining analysis**

163 **Trypan blue staining:** Potato leaves inoculated with *P. infestances* for 5 days were
164 soaked in Trypan blue solution (0.25 mg/mL) for 12 hours and then boiled to
165 decolorize in the solution of lactophenol and absolute alcohol (volume ratio 1:2).

166 **DAB staining:** Potato leaves inoculated with *P. infestances* for 0, 6, 12 and 24 hours
167 were soaked in the DAB (diaminobenzidine, Sigma) solution (1 mg/mL) for 8 hours
168 and then boiled to decolorize as mentioned above.

169 **Aniline blue staining:** Potato leaves inoculated with *P. infestances* for 7 days were
170 fixed in FAA solution (volume ratio of formaldehyde: acetic acid: absolute alcohol:
171 water was 2:1:9:8), then leaves were soaked in 0.01% Aniline blue solution overnight
172 and boiled to decolorize in solution of lactophenol and absolute alcohol (volume ratio
173 1:2). 0.02% fluorescent dye calcofluor was added onto decolorized leaves and
174 samples were observed by positive microscope Zeiss Axioskop 40 (Carl Zeiss AG) in
175 the ultraviolet excitation module (FT-395 nm, LP-42 nm). Callose was in
176 yellow-green while sporangia and mycelium were in blue-purple.

177 **Preparation of transmission electron microscope samples**

178 Potato leaves inoculated with *P. infestances* for 0, 12 and 24 hours were sampled for
 179 transmission electron microscope observation. The leaves around the inoculation site
 180 were cut into 0.1 cm × 0.2 cm pieces, and then those pieces were evacuated in 4%
 181 glutaraldehyde phosphate buffer (0.1 mol/L, PH 6.8). Vacuumized leaf samples were
 182 fixed in 4% glutaraldehyde for 3 hours, and then samples were rinsed by the
 183 phosphate buffer solution (pH6.8) for 5 times, 15 minutes each. Samples were then
 184 fixed in 1% osmic acid phosphate buffer (0.1 mol/L, PH 6.8) for 2 hours and rinsed
 185 by phosphoric acid buffer solution (pH6.8) for 4 times, 15 minutes each time. After
 186 that, the samples were dehydrated by 30%, 50%, 70%, 80%, 90% and 100% ethanol
 187 step by step, 30 minutes in each step. Then samples were dehydrated by 100%
 188 acetone for 3 times, 30 minutes each time before embedded with epoxy resin Epon812
 189 and polymerized at 30 °C for 24 hours and 60 °C for 48 hours. Embedded sample
 190 blocks were positioned by glass cutter via semi thin section and positioned slice was
 191 made into ultra thin section by the diamond cutter. Slices were set onto the copper
 192 screen, and stained in 2% uranyl acetate for 10 minutes, following with staining in 2%
 193 lead citrate for 5 minutes. Slices were rinsed in distilled water and dried on a filter
 194 paper, and at last slices were observed and photographed via the transmission electron
 195 microscope JEM-1230 (JEOL).

196 **Results**

197 **Isolation of the *StPOPA* gene in potato and cluster analysis of its homologous**
 198 **proteins**

199 Cloning of the full-length cDNA of the *StPOPA* gene was done by electronic
200 cloning (Du et al., 2013). The *StPOPA* gene has a length of 1092 bp encoding 363
201 amino acids. Cluster analysis of the *StPOPA* gene and its homologous genes in potato
202 and other plants (data from NCBI database) was carried out, and it was indicated that
203 three genes in tomato (gi|723674143, gi|225321568 and gi|19359) had the highest
204 similarity with the *StPOPA* gene (Fig 1A). The suberization-associated anionic
205 peroxidase 2-like gene (gi|565360827), with the name *StTAP2* in the database, from
206 potato clustered closely to the *StPOPA* gene was taken as a homologous gene of the
207 *StPOPA* gene.

208 **Protein structure prediction and subcellular localization of the StPOPA**

209 The signal peptide prediction result by SignalP 4.1 Server database
210 (<http://www.cbs.dtu.dk/services/SignalP/>) showed that the StPOPA protein contained a
211 significant signal peptide with the possibility of 0.790 (Fig 1B) and the subcellular
212 localization prediction by the TargetP 1.1 Server database
213 (<http://www.cbs.dtu.dk/services/TargetP/>) showed that the StPOPA protein might be
214 located in the secretory pathway. To clarify the subcellular localization of the StPOPA
215 protein in plant cells, we constructed a vector in which the StPOPA protein and GFP
216 protein were fusion expressed. The subcellular localization of the StPOPA::GFP
217 protein was observed by confocal after transforming *N. Benthamiana* for 36 hours. It
218 was indicated that the StPOPA protein was mainly located in the cell membrane (CM),
219 but we could vaguely observe GFP signal in the endoplasmic reticulum (ER), which
220 was diffusely distributed around the nucleus. Moreover, we could even observe some

221 small bright spots sporadically in the cell membrane, which were probably secretory
222 vesicles (SV) (Fig 1C). These results suggested that the StPOPA protein was probably
223 a secretory protein, and these results were very consistent with the characteristics of
224 an enzyme.

225 **Expression profiling of *StPOPA* gene**

226 In order to study the tissue expression profile and biotic and abiotic factors
227 induced expression profile of the *StPOPA* gene, we detected the expression level of
228 the *StPOPA* gene in different tissues of potato and the expression level of the *StPOPA*
229 gene after *P. infestans* infection, mechanical damage and plant hormone treatment. It
230 was shown that *StPOPA* gene could be detected in all the tissues with varied levels.
231 The highest expression was found in the upper stems while the lowest in the tubers
232 (Fig 2A).

233 With regard to figure out whether the *StPOPA* gene was induced by the infection
234 of *P. infestans*, we detected the expression level of the *StPOPA* gene in E3 after
235 infection by two different *P. infestans* races, Ljx18 and 14-2 which performed
236 incompatible and compatible interaction with E3, respectively. The results showed
237 that the two races could both slightly induce the expression of *StPOPA* in the first 12
238 hours, followed by a rapid rise in transcriptional level at 24 hours inoculated with
239 Ljx18 and at 48 hours with 14-2 while they both induced the highest expression of the
240 *StPOPA* gene at 60 hours (Fig 2B). Our results demonstrated that *P. infestans* infection
241 could induce an up-regulated expression of the *StPOPA* gene with an earlier response
242 to incompatible race than compatible one, suggesting that the *StPOPA* gene may play

243 a positive role against late blight in a relative late stage of the disease development.

244 The expression level of the *StPOPA* gene didn't change significantly at the early
245 stage after mechanical damage (Fig 2C), but the expression level of the *StPOPA* gene
246 increased rapidly after 24 hours, implying that the *StPOPA* gene may have function in
247 response to cell damage. Moreover, application of jasmonate (MeJA) and ethylene
248 (ET) could activate the *StPOPA* gene to a high level in the first 3 hours but no
249 significant effects were detected for salicylic acid (SA) (Fig 2D), speculating that the
250 expression of the *StPOPA* gene could be regulated by the JA/ET signal pathway.

251 **Function dissection of *StPOPA* in resistance to *P. infestans***

252 In order to find out whether the *StPOPA* gene has a valid function in resistance to
253 *P. infestans*, the function complementation test was conducted by overexpressing and
254 silencing of the *StPOPA* gene, as well as simultaneously silencing *StPOPA* and its
255 homologous gene *StTAP2* in potato variety E3. Selected transgenic lines of each
256 transformation were illustrated in Fig. 3A-3C.

257 For evaluating the effects of the *StPOPA* gene on lesion expansion area, detached
258 leaves of transgenic plants together with wild-type E3 and E3 transformed with the
259 35S:GUS vector were inoculated with *P. infestances*. It was indicated that leaf lesion
260 area of overexpression lines was significantly smaller than wild-type and E3
261 transformed with 35S:GUS, whereas no significant difference was observed for the
262 specific RNA-interference lines (Fig. 3D). However, silencing both the *StPOPA* and
263 the *StTAP2* genes (nonspecific RNA-interference lines) exhibited a significantly larger
264 lesion area than control (Fig. 3E), implying a redundant function of *StTAP2* to *StPOPA*

265 in late blight resistance.

266 **Histochemical responses of transgenic leaves to *P. infestans* infection**

267 The transgenic leaves together with control were stained using DAB to monitor
268 the accumulation of H₂O₂ in cells. The H₂O₂ could be visualized after 6 hours of the
269 inoculation. Overexpression of the *StPOPA* gene led to a higher accumulation of H₂O₂
270 while silencing the *StPOPA* gene resulted in a lower H₂O₂ abundance compared to
271 wild-type and 35S:GUS transformed controls (Fig 4A). It was interesting that
272 nonspecific interference of the *StPOPA* gene (i.e. silencing both *StPOPA* and its
273 homologous *StTAP2*) had even a lower H₂O₂ production than the specific interference
274 of the *StPOPA* gene. These results suggested that the *StPOPA* gene could transmit
275 signal by reactive oxygen species to induce the resistance response to *P. infestans*
276 infection.

277 Similar results were observed in callose in the cells as stained by aniline blue.
278 Abundant callose deposited around the point of inoculation in the leaves of
279 overexpression lines and thickness of cell wall increased visibly (Fig 4B). On the
280 contrary, no apparent accumulation of callose was observed in the leaves of specific
281 interference and nonspecific interference lines. To confirm if the biosynthesis of
282 callose was affected by the *StPOPA* gene, we tested the expression of β-1,3 glucan
283 synthase, a key enzyme for callose synthesis. The full-length sequence of potato β-1,3
284 glucan synthase gene was obtained in NCBI database (*Solanum tuberosum* callose
285 synthase 2-like, LOC102595703) and the expression level of β-1,3 glucan synthase
286 gene in leaves of all the transgenic lines at multiple time points after *P. infestans*

287 inoculation was detected by real-time quantitative PCR. From Fig 4C we could figure
288 out that the relative expression level of β -1,3 glucan synthase gene increased during
289 infection. In comparison to control, the relative expression level of the β -1,3 glucan
290 synthase gene was remarkably elevated in overexpression line after 6 hours of *P.*
291 *infestances* infection while the expression of the β -1,3 glucan synthase gene was
292 significantly repressed in silenced lines. These findings implied that the *StPOPA* gene
293 might play a key role in the process of callose formation by enhancing callose
294 formation and deposit in the cell wall, withstanding the invasion and further spread of
295 *P. infestance*.

296 The cell death around the inoculation point was detected by Trypan blue staining.
297 Thirty-six hours after *P. infestances* inoculation, blue spots on the leaves of
298 overexpression line were lighter than those of control but darker than control in the
299 leaves of silenced lines with a more severe situation in the nonspecific interference
300 line (Fig 4D). These results provided a conclusion that reinforcing the *StPOPA* gene
301 could primarily reduce the cell damage by *P. infestans*.

302 **Effect of the *StPOPA* gene on cell ultra microstructure**

303 With regard to elucidate the effects of the *StPOPA* gene on the pathogenesis of *P.*
304 *infestances* in plant cells, the ultrastructure of the leaves sampled from each
305 transgenic line after *P. infestances* inoculation was observed by electron microscopy
306 (Fig 5). As a control, in the healthy potato leaves, chloroplasts in cells were regularly
307 arranged on cytoplasmic membranes.

308 There were no much variations in all leaf samples in the first 12 hours of *P.*

309 *infestans* inoculation except that the mycelia of the pathogen extended along the cell
310 wall of host cells or the gap between adjacent host cells in the leaves of overexpressed
311 line. When the mycelia passed through cell wall of host cell, they formed haustellum
312 between cell wall and cell membrane. After 24 hours, in the cells of leaves of the
313 overexpressed line, plasmolysis was observed and much electron-dense material
314 deposited in the space between the cell wall and the cell membrane. The outer
315 membrane of chloroplasts disintegrated, the morphology of the chloroplasts gradually
316 turned round and the laminated structure of thylakoid was slightly expanded.
317 However, in the cells of leaves of specific interference line, the organelles of host
318 cells such as the nucleus, chloroplast and endoplasmic reticulum were degraded while
319 part of the complete organelle structure could still be observed. The more severe
320 situation was in the cells of leaves of nonspecific interference line, the host cells were
321 obviously necrotic, the organelles of adjacent cells were mixed together and
322 completely degraded. These results were in accordance with the finding above that the
323 *StPOPA* gene may have a function of maintaining cell integrity to reduce the damage
324 caused by *P. infestans* infection.

325 **Discussion**

326 Peroxidases are ubiquitous in plants (Milla et al., 2010) and they have a different
327 degree of substrate specificity (Leon et al., 2002) and spatio-temporal expression
328 pattern (Valério et al., 2004). The anionic peroxidase gene *StPOPA*, screened from our
329 previous potato ESTs challenged with *P. infestans* (Du et al., 2013; Li et al., 2009),
330 was proved to contribute to potato late blight resistance by complementary function

331 dissection in the present research (Fig. 3). The *StPOPA* gene is constitutively
332 expressed in potato plants, and its transcripts can be induced rapidly by jasmonic acid
333 and ethylene and little latter by wounding and *P. infestans* (Fig. 2), suggesting that the
334 *StPOPA* gene may have a function associated with cell damage in the signal pathway
335 of JA/ETH.

336 It is noticeable that co-suppression of the *StPOPA* gene and its homologous gene
337 *StTAP2* exhibited a higher resistance level to *P. infestans* than did by suppressing only
338 the *StPOPA* gene (Fig. 3D). Previous study showed that biotic and abiotic stress could
339 both induce the expression of different peroxidase isoenzymes (Navrot et al., 2006).
340 Besides, there were reports also identified the similar function of different peroxidase
341 isoforms (Reumann and Bartel, 2016). Therefore, we speculated that difference in late
342 blight resistance of specific interference of the *StPOPA* gene and nonspecific
343 interference of both the *StPOPA* and *StTAP2* genes may come from the functional
344 redundancy of these homologous genes.

345 It is well understand that oxidative burst characterized by accumulation H_2O_2 in
346 plant cells is one of the defense responses of plants against pathogen infection. Plants
347 usually generate reactive oxygen species (ROS) as signaling molecules that activate
348 various processes including pathogen defense, programmed cell death, and stomatal
349 behavior (Apel and Hirt, 2004). In the present research, we clarified that H_2O_2 was
350 predominantly formed by overexpressing the *StPOPA* gene (Fig 4A). A possible
351 quantitative correlation between the *StPOPA* gene expression and H_2O_2 abundance
352 was observed. A possible explanation could be that, as a peroxidase, *StPOPA*

353 catalyzes the formation of H₂O₂ to control the process of plant defense against the
354 invasion of *P. infestans*. It was indicated that in the process of pathogen defense
355 response, the plant simultaneously produced more ROS while decreasing its ROS
356 scavenging capacities, this led to the accumulation of ROS and activation of
357 programmed cell death (PCD) (Delledonne et al., 2001; Foyer and Noctor, 2000). Our
358 results demonstrated that severe cell death was accompanied with the suppression of
359 the *StPOPA* gene, indicating a potential mode of the *StPOPA* gene to defeat late blight
360 by enhancing H₂O₂ production for purposeful programmed cell death.

361 It was documented that, in the presence of H₂O₂ and phenolic substrates,
362 peroxidases could act in the peroxidatic cycle and were engaged in the synthesis of
363 lignin and other phenolic polymers (Apel and Hirt, 2004). When the pathogen infects,
364 the cell wall rehabilitation and reinforcement are the first physiological barrier to
365 resist the pathogen invasion. The cell wall would accumulate a lot of callose and
366 lignin for cell wall lignification and the formation of the mastoid. In our study,
367 overexpression of the *StPOPA* gene could promote the formation of callose and
368 possibly other cell wall sediments and the deposition of electron-dense materials (Fig
369 5). This finding was further confirmed by a higher expression level of the β-1,3
370 glucan synthase gene in the *StPOPA* gene overexpressed plants than control and the
371 *StPOPA* gene silenced plants (Fig. 4C). Synthesis of callose possibly by enhancing
372 β-1,3 glucan synthase was thus considered an effective way for the *StPOPA* gene to
373 defense against the infection of *P. infestans*. This process led to a significant inhibition
374 of the pathogen growth and expansion in the host cells of the *StPOPA* gene

375 overexpressed plants (Fig. 4D).

376 And a little bit more, our results showed that overexpression of the *StPOPA* gene
377 was correlated with a decrease in the resistance and a marked increase in ROS and
378 callose synthesis, so we could conclude that overexpression of the *StPOPA* gene
379 increased potato resistance to *P. infestans*. It was reported that the apoplastic oxidative
380 burst generated by peroxidases was an important component of PTI and it could
381 contribute to DAMP-elicited defenses such as callose synthesis (Daudi et al., 2012).
382 However, overexpression of anionic peroxidases was known to affect plant
383 development in some cases, especially root growth (Lagrimini et al., 1997). So there
384 was a possibility that the generation of ROS in the *StPOPA* overexpression lines might
385 just a consequence of the deregulation of the apoplastic peroxidases synthesis. And it's
386 highly likely that the primary role of anionic peroxidases in the resistance to
387 pathogens, was to use rapid hydrogen peroxide to cross-link the different phenolic
388 monomers occurring in lignin or in the phenolic domain of suberin (Quiroga et al.,
389 2000). In order to clarify the exact mechanism of the *StPOPA* gene, more work
390 remained to be done in our future research.

391 In conclusion, our present research revealed that the *StPOPA* gene contributed to
392 potato resistance against *P. infestans*. On the one hand, the *StPOPA* gene could
393 promote H₂O₂ burst and cell necrosis, restraining the infection of pathogenic bacteria
394 and expansion of mycelium. And on the other hand, enhanced peroxidase activity may
395 catalyze the synthesis of callose, thus preventing the invasion of pathogenic bacteria
396 and its expansion in the host cells.

397 **Supplementary data**

398 Supplementary Table S1. Primers used in this study.

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546 **Figure 1. Cluster analysis, signal peptide prediction and subcellular localization**

547 **of the StPOPA protein.** A, Cluster analysis of the StPOPA homologous protein in
548 potato and other plants. Red box represents the StPOPA clone in this study. B, Signal
549 peptide prediction of the StPOPA protein in SignalP 4.1 Server. C,
550 Subcellular localization of the StPOPA protein in epidermal cells of *N. benthamiana*.
551 Bright spots in red circles were possible secretory vesicles. CM, cell membrane. ER,
552 endoplasmic reticulum. N, nucleus. Bar, 20 μ M.

553

554 **Figure 2. Tissue expression profile and induced expression profile of the *StPOPA***

555 **gene.** A, Relative expression level of the *StPOPA* gene in representative tissues of
556 potato. B, Relative expression level of the *StPOPA* gene in potato leaves after infected
557 by *P. infestans*. C, Relative expression level of the *StPOPA* gene in potato leaves after
558 mechanical damage. D, Relative expression level of the *StPOPA* gene in potato leaves
559 after treated by hormones.

560

561 **Figure 3. Resistance to late blight of the *StPOPA* gene overexpression and**

562 **RNA-interference transgenic plants.** A, Relative expression level of the *StPOPA*
563 gene in leaves of overexpression plants. B, Relative expression level of the *StPOPA*
564 gene in leaves of specific interference plants. C, Relative expression level of the
565 *StPOPA* and *StTAP2* genes in leaves of nonspecific interference plants. D, Lesion area
566 in leaves of the *StPOPA* gene overexpression and specific RNA-interference
567 transgenic plants after *P. infestans* inoculation for 3, 4 and 5 days. Error bars

568 indicate the standard deviation of 3 replicates. “*” indicates significant differences at
 569 $p < 0.05$, “***” indicates extremely significant differences at $p < 0.01$. E, Lesion area in
 570 leaves of nonspecific RNA-interference transgenic plants after *P. infestans*
 571 inoculation for 3, 4 and 5 days. Error bars indicate the standard deviation of 3
 572 replicates. “*” indicates significant differences at $p < 0.05$, “***” indicates extremely
 573 significant differences at $p < 0.01$.

574

575 **Figure 4. Accumulation of H₂O₂ and callose and cell death in leaves of the**
 576 ***StPOPA* gene overexpression (OE), specific silencing (SRI) and non-specific**
 577 **silencing (NSRI) plants. A, DAB staining of H₂O₂ for 0, 6, 12 and 24 hours after *P.***
 578 ***infestans* inoculation. Bars, 200 μ m. B, Aniline blue staining of callose 5 days after *P.***
 579 ***infestans* inoculation. Bars, 50 μ m. C, Relative Expression of β -1, 3 glucan synthase**
 580 **gene encoding the enzyme for biosynthesis of callose. D, Trypan blue staining of dead**
 581 **cells 36 hours after *P. infestans* inoculation. Bars, 200 μ m. Wild type E-Potato 3 and**
 582 **35S:GUS were taken as controls.**

583

584 **Figure 5. Transmission electron micrographs of leaves of the *StPOPA* gene**
 585 **overexpression (OE) and RNA-interference (SRI and NSRI) transgenic plants**
 586 **after *P. infestans* inoculation for 0, 12 and 24 hours. CH, chloroplast; CW, cell wall;**
 587 **CY, cytomembrane; EM, electron-dense material; HY, hyphae; IH, intercellular**
 588 **hyphae; SG, Starch granule.**

589

590 **Fig1**

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592 **Fig2**

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595 **Fig3**

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598 **Fig4**

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601 **Fig5**

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