1 **The effector protein CgNLP1 of *Colletotrichum gloeosporioides* from *Hevea***
2 ***brasiliensis* disrupts nuclear localization of necrosis-induced transcription factor**
3 **HbMYB8-like to suppress plant defense signaling**

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14 **Summary**

- 15 • *Colletotrichum gloeosporioides* is the dominant causal agent of rubber tree
16 anthracnose and leads to serious loss of natural rubber production. Fungi secrete
17 numerous effectors to modulate host defense systems. Understanding the molecular
18 mechanisms by which fungal effectors regulate plant defense is of great importance
19 for the development of novel strategies for disease control.
- 20 • Here, we identified an NLP effector gene, *CgNLP1*, which contributed to

virulence of *C. gloeosporioides* to rubber tree. Transient expression of CgNLP1 in the leaves of *Nicotiana benthamiana* induced ethylene production in plants. Ectopic expression of CgNLP1 in Arabidopsis significantly enhanced the resistance to *Botrytis cinerea* and *A. brassicicola*.

- CgNLP1 was shown to target a R2R3 type transcription factor HbMYB8-like in rubber tree, which localized on nucleus and induced necrosis in *N. benthamiana*. CgNLP1 disrupted nuclear accumulation of HbMYB8-like and suppressed necrosis induced by HbMYB8-like mediated SA signal pathway.

- This work suggested a strategy whereby *C. gloeosporioides* exploited CgNLP1 effector to suppress host defense to facilitate infection by disrupting the subcellular compartment of a host defense regulator HbMYB8-like.

Keywords: *Colletotrichum gloeosporioides*; CgNLP1; pathogenic mechanism; *Hevea brasiliensis*; HbMYB8-like

Introduction

Colletotrichum causes anthracnose on a wide variety of woody plants in tropical, subtropical and temperate climates (Liang et al., 2021). *C. gloeosporioides* is the dominant causal agent of rubber tree anthracnose and leads to serious loss of natural rubber production (Liu et al., 2018). To successfully infect and cause disease, phytopathogenic fungi need to form intimate associations and maintain constant communication with a susceptible host, and this communication can be achieved through the proteins, enzymes and metabolites secreted by phytopathogenic fungi (Heard et al., 2015). Effector proteins are small cysteine-rich proteins secreted by pathogens and play roles in virulence and the interaction between plant and pathogens. According to the innate immunity theory, plants have evolved two strategies to detect pathogens: one is the recognition of conserved microbial elicitors called pathogen associated molecular patterns (PAMPs) by receptor proteins called pattern recognition receptors (PRRs) on the external face of the host cell, which leads to PAMP-triggered

immunity (PTI); another one is the recognition of pathogen virulence molecules called effectors by plant intracellular receptors called R protein, which leads to effector-triggered immunity (ETI) (Dodds & Rathjen, 2010).

The necrosis- and ethylene-inducing protein 1 (Nep1)-like proteins (NLPs) are an important effector family and named after the necrosis and ethylene-inducing protein (NEP1) firstly identified from culture filtrate of *Fusarium oxysporum* f.sp. *erythroxyli* (Bailey, 1995; Chen et al., 2018). NLPs widely distributed in oomycetes, fungi and bacteria, and shared a conserved necrosis-inducing phytophthora protein (NPP1) domain, typically containing a GHRHDWE heptapeptide motif which was crucial for toxicity (Gijzen & Nürnberger, 2006; Lenarčič et al., 2017). Based on the molecular structures and sequence comparison analysis, NLPs were divided into three phylogenetic group types: type I, type II and type III (Oome et al., 2014; Levin et al., 2019; Seidl & Ackerveken, 2019). Type I NLPs contained a conserved disulfide bond and were found predominately in plant microorganisms. Compared with type I, type II had a second conserved disulfide bond and an additional putative calcium-binding domain. Type III NLPs were different from the other types in the amino acid sequence of N- and C-terminal portion, and there is still very little experimental data available (Oome et al., 2014; Lenarčič et al., 2017). Based on the ability to induce necrosis, NLPs were divided into two groups (Schumacher et al., 2020). Group one was cytotoxic NLP proteins and group two was non-cytotoxic NLP proteins (Amsellem et al., 2002; Santhanam et al., 2013).

The members of group one as their names suggested were best known as a virulence factor for inducing necrosis and ethylene production in plant leaves (Bailey, 1995; Oome et al., 2014; Levin et al., 2019; Seidl & Van den Ackerveken, 2019). MoNEP1, MoNLP2 and MoNEP4, three NLP proteins from the hemibiotrophic plant pathogenic fungus *Magnaporthe grisea*, induced cell death and the production of reactive oxygen species in *N. benthamiana* (Fang et al., 2017). BcNEP1 and BcNEP2, two paralogous NLPs from the necrotrophic plant pathogenic fungus *Botrytis cinerea*, caused necrosis

78 in all dicotyledonous plant species tested (Schouten et al., 2008). Normally, the
79 cytotoxic NLP proteins were expressed during the switch from biotrophic to
80 necrotrophic lifestyle in hemibiotrophic pathogens (Alkan et al., 2015). It had been
81 proved that NLP-induced cell death was an active, light-dependent process that
82 required HSP90 and interacts with a target site on extracytoplasmic side of dicot plant
83 plasma membranes (Qutob et al., 2006). Biochemical analyses had revealed that the
84 target of NLP on plant membrane was sphingolipid-glycosyl inositol phosphorylated
85 ceramide (GIPC) which consisted of an inositol phosphoceramide and a head group
86 consisting of glucuronic acid and a variable number and form of terminal hexoses.
87 When NLPs bind to the head group of GIPC in monocotyledons, the three terminal
88 hexoses prevented NLPs from inserting into the lipid bilayers of cell membranes,
89 while the GIPC head of dicotyledons only had two terminal hexoses, allowing NLPs
90 to insert into cell membranes and causing cell necrosis (Van den Ackerveken, 2017).
91 Recent research results showed that a plant-derived LRR-only protein NTCD4
92 promotes NLP-triggered cell death and disease susceptibility by facilitating
93 oligomerization of NLP in Arabidopsis (Chen et al., 2021).
94 Group two were non-cytotoxic NLP proteins which were often expressed during very
95 early stages of the infection or keep at rather low levels during the whole course of
96 infection (Cabral et al., 2012; Dong et al., 2012; Schumacher et al., 2020). These
97 non-cytotoxic NLPs also acted as triggers of plant innate immune responses,
98 including posttranslational activation of mitogen-activated protein kinase activity,
99 deposition of callose, production of nitric oxide, reactive oxygen intermediates,
100 ethylene, the phytoalexin camalexin and cell death (Fellbrich et al., 2000; Kanneganti
101 et al., 2006; Qutob et al., 2006; Rauhut et al., 2009; Villela-Dias et al., 2014; Seidl &
102 Van den Ackerveken, 2019). Ten different noncytotoxic NLPs (HaNLPs) from
103 biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* did not cause
104 necrosis, but acted as potent activators of the plant immune system in *Arabidopsis*
105 *thaliana*, and ectopic expression of HaNLP3 in Arabidopsis enhanced the resistance
106 to *H. arabidopsidis* and activated the expression of a large set of defense-related
107 genes (Oome et al., 2014). Moreover, it was also reported that multiple cytotoxic

NLPs carried a motif of 20 amino acid residues (nlp20), and nlp20 could trigger PTI by binding in vivo to a tripartite complex RLP23-SOBIR1-BAK1 (Böhm et al., 2014; Albert et al., 2015). Ectopic expression of RLP23 in potato (*Solanum tuberosum*) enhanced immunity to *Phytophthora infestans* and *Sclerotinia sclerotiorum* (Albert et al., 2015).

In *Colletotrichum*, six NLP homologs were identified in the *C. higginsianum* genome. Of them, ChNLP1 induced cell death when expressed transiently in *Nicotiana benthamiana* and was expressed specifically at the switch from biotrophy to necrotrophy, whereas ChNLP3 lacks necrosis-inducing activity and was expressed in appressoria before penetration and (Kleemann et al., 2012). An effector NLP1 from *C. orbiculare* induced necrosis in *N. benthamiana* and also possessed MAMP sequence called nlp24 which triggered the ROS accumulation in leaf discs of Arabidopsis (Azmi et al., 2017). In this study, an NLP effector protein identified in *C. gloeosporioides* was named as CgNLP1 which contributed to virulence of *C. gloeosporioides* to rubber tree and enhanced the resistance of Arabidopsis to *B. cinerea*. A R2R3-type transcription factor HbMYB8-like, which localized on nucleus and induced necrosis, was identified as the target of CgNLP1 in rubber tree, and CgNLP1 disrupted the nuclear translocation of HbMYB8-like and inhibits HbMYB8-like-induced cell death mediated by SA signaling. Our results provide new insights into the molecular mechanisms of interaction between rubber tree and *C. gloeosporioides* mediated by CgNLP1.

Materials and methods

Biological materials and growth conditions

Colletotrichum gloeosporioides strain was isolated from the leaves of *Hevea brasiliensis* with anthracnose. *Botrytis cinerea* and *Alternaria brassicicola* courtesy of Tesfaye's lab. All fungal strains were grown on potato dextrose agar (PDA) at 28°C in the dark. *Hevea brasiliensis* (Reyan 7-33-97) was grown on soil at 28°C. *Arabidopsis thaliana* columbia ecotype and *Nicotiana benthamiana* were grown on soil under

137 fluorescent light ($200 \mu\text{E}\cdot\text{m}^2\cdot\text{s}^{-1}$) at 22°C with 60% RH and a 12-h-light/12-h-dark
138 cycle.

139 **RNA Isolation, cDNA Synthesis, PCR amplification and qRT-PCR**

140 Fungal total RNA was extracted using CTAB-LiCl method (Yang J et al., 2020). Plant
141 total RNA were extracted according to the instruction of the polysaccharide
142 polyphenol plant total RNA extraction kit (Tiangen: DP441). The contaminating DNA
143 was eliminated using RNase-free DNase and the first-strand cDNA was synthesized
144 using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher). Quantitative
145 RT-PCR analysis was performed using ChamQ Universal SYBR qPCR Master Mix
146 (Vazyme Biotech: Q711-02) with the LightCycler 96 System (Roche). The *N.*
147 *tabacum actin-7* (*NtActin 7*) and *H. brasiliensis* 18S rRNA (*Hb18S*) were used as an
148 endogenous control for normalization. The primers used for quantitative RT-PCR and
149 PCR amplification were list in Supplemental Table 1. Relative expression levels of
150 target genes were estimated using the $2^{-\Delta\Delta\text{Ct}}$ method.

151 **Sequence analysis of CgNLP1 and HbMYB8-like**

152 The amino acid sequences were deduced by DNAMAN software. Predictions of
153 signal peptides were performed online by SignalP 5.0 analysis tool
154 (<http://www.cbs.dtu.dk/services/SignalP/>). The conserved domains were predicted
155 using SMART website (<http://smart.embl-heidelberg.de/>). The multiple alignments of
156 amino acid sequences were performed using ESPript 3.0
157 (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>) and GeneDoc 2.7.0. The bootstrap
158 neighbor-joining phylogenetic tree was constructed using Clustal X 2.0 and MEGA
159 7.0.

160 **Generation of CgNLP1 knockout and complementary mutants**

161 Based on the diagram of *CgNLP1* knockout vector, the 5' and 3' flanking region of
162 *CgNLP1* were amplified from genomic DNA and ligated into the vector pCB1532
163 carrying the acetolactate synthase gene (SUR) cassette conferred resistance to
164 chlorimuron ethyl (a sulfonylurea herbicide). For the complementation vector, the
165 open read frame of *CgNLP1* fused with the 3 X FLAG coding sequence was cloned
166 into the vector harboring the promoter of *ToxA*, the terminator of *nos* and the

167 hygromycin phosphotransferase gene (HPH) (Figure S2a). Fungal transformation was
168 carried out as described in our previous work (Wang et al., 2018). The mutants were
169 analyzed by PCR analyses. The primers used for *CgNLP1* amplifying and mutant
170 diagnosis were list in Supplemental Table 1.

171 **Ethylene production assays**

172 The tobacco leaves transiently expressing *CgNLP1* was collected post *Agrobacterium*
173 injection every 12 hours for 3 days and kept in sample bottles for 3 days. 0.5 mL of
174 ethylene released from leaves then was moved to syringe for ethylene contents
175 assayed using a portable ethylene analyzer (GC-FID 8890, Agilent, USA).
176 Ethylene standards (99.99% purity) was used for standard curve construction.

177 **Construction of *CgNLP1* overexpression lines in Arabidopsis**

178 The vector pER8-*CgNLP1*-FLAG was constructed with an estradiol-inducible
179 promoter and then transformed into *Agrobacterium tumefaciens* GV3101.
180 *Agrobacterium*-mediated flower dip method was used for Arabidopsis transformation
181 of *CgNLP1*. T1 transgenic lines were screened with 30 mg/L hygromycin and Western
182 Blot was used to confirm the positive transgenic lines after estradiol treatment.
183 Appropriate total proteins from different lines were resolved on 10 % polyacrylamide
184 gels and transferred to a nitrocellulose membrane (Bio-Rad). Anti-FLAG monoclonal
185 antibody (1:1000, ab125243; Abcam) was used as primary antibody to detect the
186 expression of *CgNLP1* in transgenic lines. Horseradish peroxidase-conjugated
187 anti-mouse antibody was used as the secondary antibody, and the signal was detected
188 using the ECL western detection kit (RPN2232; GE Healthcare).

189 **Disease assays**

190 For the pathogenicity test of *C. gloeosporioides* to rubber tree, Conidia were
191 harvested from mycelium grown on PDA (potato dextrose agar, Difco) medium for 10
192 days in a 28°C incubator and resuspended in a solution of PD (potato dextrose broth,
193 Difco) liquid medium to a final concentration of 2×10^5 conidia/mL. Then 10 µL of
194 the conidial suspensions were inoculated onto the wounded rubber tree variety
195 7-33-97 leaves at “light green” stage. The inoculated leaves were kept in a moist
196 chamber at 28 °C under natural illumination for 4 days, and the disease symptoms

197 were scored.

198 *B. cinerea* and *A. brassicicola* disease assay were performed on detached leaves by
199 drop inoculation. Both strains were cultured in 2×V8 solid medium and incubated at
200 22°C. Conidia of *B. cinerea* were collected and suspended in 1% Sabouraud maltose
201 broth buffer (Difco) containing 0.05% (v/v) Tween-20 and the conidia density was
202 adjusted to 2.5×10^5 spores/mL before inoculation. Conidia of *A. brassicicola* were
203 collected and suspended in water containing 0.05% (v/v) Tween-20 and the conidia
204 density was adjusted to 5×10^5 spores/mL before inoculation. In both cases, the
205 droplets of 5 µL spore suspension above were inoculated on 4-week-old Arabidopsis
206 leaves. The inoculated plants were kept under a transparent cover to maintain high
207 humidity. The lesion diameters were measured to assess the levels of plant disease
208 resistance. Each treatment contained three replicates of 9 leaves and the entire
209 experiment was repeated three times.

210 **Subcellular localization and bimolecular fluorescence complementation (BiFC)** 211 **assays**

212 For Subcellular localization of HbMYB8-like, the coding sequence of *HbMYB8-like*
213 was inserted into the transient expression vector 35S-MCS-mScarlet to generate
214 recombinant plasmid HbMYB8-like-RFP. The vector MEIL-RFP was used as marker
215 vector for plasma membrane and nuclear localization (Guy et al., 2013). For BiFC
216 assay, the coding sequences of *CgNLP1* and *HbMYB8-like* were inserted into
217 pSPYCE-YFP^C and pSPYNE-YFP^N respectively to generate recombinant plasmids
218 pSPYCE-*CgNLP1*-YFP^C and pSPYNE-*HbMYB8-like*-YFP^N. The above constructs
219 were verified by sequencing and then introduced into Agrobacterium strain GV3101,
220 respectively. The Agrobacterium carrying HbMYB-like-RFP was expressed in
221 *Nicotiana benthamiana* leaf tissue by agroinfiltration for Subcellular localization.
222 pSPYCE-*CgNLP1*-YFP^C and pSPYNE-*HbMYB8-like*-YFP^N was co-expressed in
223 *Nicotiana benthamiana* leaf tissue for BiFC assay. The fluorescence distribution was
224 observed with a laser confocal microscope (Leica TCS SP8).

225 **Phytohormones treatment**

226 Seedlings of Reyan7-33-97 were treated with 5 mM salicylic acid (SA), 1 mM methyl

jasmonate (MeJA), 0.5 mM ethephon (ET) and placed in 25°C green house (Yang et al., 2020). After 0, 12, 24, and 48 hours following treating, leaves from seedlings were collected and quickly frozen in liquid nitrogen and then stored in a -80°C refrigerator for RNA extraction. Two leaves from each seeding were picked and three seedlings were pooled as one biological replicate. Each seeding was harvested only once.

Yeast Two-hybrid Screens

Yeast two-hybrid assays were performed with Matchmaker™ Gold Yeast Two-Hybrid System according to the manufacturer's instructions (Clotech: No.630489). The coding sequence of *CgNLP1* was amplified and cloned into pGBKT7 to generate DNA binding domain bait protein fusion. The cDNA libraries of *Hevea brasiliensis* was constructed according to the manufacturer's instructions (Clotech: PT4085-1). Interacting proteins were selected for selective medium lacking His, Leu, Trp and Ade. The putative interactors were then tested by assaying for the lacZ reporter gene activation as described in the Clotech protocol. The plasmids from the positive clones were then isolated and reintroduced into the original yeast bait and control bait strains to verify interaction.

GST Pull-Down Assays

The Prokaryotic expression vectors pCold™ TF-CgNLP1 and pGEX-HbMYB8-like were constructed and transferred into *E. coli* BL21 (DE3) respectively. The expression of the fusion proteins was performed as described in the product manuals (Beyotime Biotechnology: P2262). The cell lysate supernatants containing GST-HbMYB8-like and His-CgNLP1 fusion protein were incubate with GST binding gels at 4°C for overnight, and the supernatants containing GST + His-CgNLP1 as the control. The pull-down reactions were analyzed by SDS-PAGE followed by Western Blot using anti-His (M20001; Abmart) and anti-GST (ab111947; Abcam) antibodies.

Trypan blue staining

Four-week-old tobacco leaves were infiltrated with *Agrobacterium tumefaciens* GV3101 harboring empty vector pEGAD-eGFP and recombinant plasmid pEGAD-*CgCPI*-eGFP, respectively. Leaves were harvested 3h after infiltration and stained for cell death using Trypan Blue Staining Cell Viability Assay Kit (Beyotime Institute of

Biotechnology, Haimen, China). For destaining, the leaf samples were boiled in bleaching solution (ethanol: acetic acid: glycerol=3:1:1) for 15 minutes.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics v.25. Differences at $P < 0.05$ were considered as significant.

Results

CgNLP1 was a type I NLP candidate effector

Based on the genome sequencing of *C. gloeosporioides* from *hevea brasiliensis*, the genes encoding extracellular secretory protein were predicted and one of them, named as *CgNLP1*, was amplified by RT-PCR. The open reading frame (ORF) of *CgNLP1* was 729 bp encoding a protein of 243 aa with two cysteine residues and a signal peptide (1-18aa) at its N-terminal (Figure S1). The amino acid sequences of *CgNLP1* were aligned with some NLP proteins identified in fungus, oomycete and bacteria (Figure 1a). The alignment showed that *CgNLP1* contained a typical NPP1 domain with a heptapeptide motif SHRHDWE, and had the highest homology with NLP protein from *verticillium dahliae*. The different types of NLP proteins identified in fungal species including *CgNLP1* were used to generate a Neighbour joining tree (Figure 1b). Phylogenetic tree analysis revealed that *CgNLP1* was clustered in the same branch with other type I NLP proteins. These results suggested that *CgNLP1* encoded a type I NLP effector protein.

CgNLP1 contributed to the virulence of *C. gloeosporioides* to rubber tree

The *CgNLP1* knockout mutant ($\Delta CgNLP1$) was obtained through gene homologous recombination technology and its complementary strain (Res- $\Delta CgNLP1$) was generated by introducing *CgNLP1* into $\Delta CgNLP1$ (Figure S2). The detached leaf inoculation assay of $\Delta CgNLP1$ and Res- $\Delta CgNLP1$ on rubber tree was performed to explore the contribution of *CgNLP1* to the pathogenicity of *C. gloeosporioides*. Results showed that typical necrotic lesions were observed in the leaves inoculated with WT, $\Delta CgNLP1$ and Res- $\Delta CgNLP1$ (Figure 2a). At 4 dpi, the lesion size caused

286 by $\Delta CgNLP1$ was significant smaller than that caused by WT, and there was no
287 significant difference in the lesion size caused by Res- $\Delta CgNLP1$ compared with the
288 WT (Figure 2b). These results indicated that the loss of *CgNLP1* resulted in reduced
289 pathogenicity of *C. gloeosporioides*, suggesting the contribution of *CgNLP1* to the
290 virulence of *C. gloeosporioides* to rubber tree.

291 ***CgNLP1* induced ethylene production but not cell death**

292 To determine the necrosis and ethylene inducing ability of *CgNLP1* in plants, tissue
293 necrosis observation and the ethylene content determination were performed in the
294 tobacco leaves transiently expressing *CgNLP1* by agroinfiltration. No obvious tissue
295 necrosis was observed in the infiltration area of tobacco leaves within 3 days post
296 infiltration and trypan blue staining results showed no obvious difference between the
297 leaves infiltrated with *A. tumefaciens* GV3101 harboring *CgCPI* gene and empty
298 vector (Figure S3). The ethylene content in the leaves expressing *CgCPI* was
299 significantly higher than that in the leaves expressing empty vector (Figure 2c), and
300 the expression levels of ACO and ACS, two key enzymes of ethylene synthesis, were
301 significantly higher in leaves expressing NLP than that in control (Figure 2d and 2e).
302 These data suggested that *CgNLP1* induced ethylene production in plant but not
303 necrosis and cell death.

304 **Ectopic expression of *CgNLP1* enhanced plant disease resistance**

305 *CgNLP1* transgenic Arabidopsis plants driven by estradiol-induced promoter were
306 generated to explore the roles of *CgNLP1* on plant disease resistance (Figure S4).
307 We next studied the possible function of *CgNLP1* in resistance against a fungus
308 pathogen by analyzing disease phenotypes of the different *CgNLP1* lines to *B. cinerea*
309 and *A. brassicicola* inoculation. Four-week-old plants were challenged with a
310 normally virulent strain of *B. cinerea* and *A. brassicicola* overexpression of *CgNLP1*
311 resulted in increased growth of the fungus and enhanced development of disease
312 symptoms and that disruption of *CgNLP1* led to decreased growth of the fungus and
313 reduced development of disease symptoms (Figure 3a, 3b and 3c). Therefore, the
314 Arabidopsis plants overexpressing *CgNLP1* significantly enhanced the resistance to *B.*
315 *cinerea* and *A. brassicicola*, these results implied that exogenous expression of

316 *CgNLP1* could improve plant disease resistance

317 ***CgNLP1* targeted rubber tree transcription factor HbMYB8-like**

318 In order to elucidate the pathogenic mechanism of *C. gloeosporioides* on rubber tree,
319 *CgNLP1*-associated proteins were identified from a cDNA library of rubber tree
320 leaves by yeast two-hybrid screening using the full-length *CgNLP1* as bait. After
321 initial screening, 25 positive clones were sequenced and five of them were chosen for
322 candidates. After verification, it is found that four of them had strong self-activation
323 and only one of them, named as HbMYB8-like, which self-activation could be
324 inhibited by adding an appropriate concentration of Aba. The *HbMYB8-like* gene was
325 amplified by RT-PCR and verified by sequencing. The result showed that the
326 full-length cDNA of *HbMYB8-like* gene was 1183 bp with a 903 bp ORF encoding
327 300 amino acids. HbMYB8-like protein contained two SANT domains which were
328 typical features of MYB transcription factors. The results of multiple sequence
329 alignment (Figure S5) and phylogenetic tree (Figure S6) showed that HbMYB8-like
330 protein had typic adjacent repeats R2R3 and clusters with R2R3-MYB type MYB
331 transcription factors from other plants, suggesting that HbMYB8-like belonged to the
332 R2R3 type MYB transcription factors.

333 The interaction of *CgNLP1* and full length HbMYB8-like was preliminarily verified
334 through yeast two-hybrid (Figure 4a). Then Pull-down and BiFC assay were
335 performed to further verify the interaction between *CgNLP1* and HbMYB8-like in
336 vitro. In pull down assay, His-tagged *CgNLP1* and GST-tagged HbMYB8-like protein
337 were expressed in *Escherichia coli* BL21 (DE3) respectively. The supernatant
338 containing GST-tagged HbMYB8-like proteins were precipitated using anti-GST
339 beads after co-incubation with supernatants containing His-tagged *CgNLP1* and His
340 protein only respectively, and the precipitates were detected using anti-His and
341 anti-GST antibodies. It was found that only His-tagged *CgNLP1* could be pulled down
342 by GST-tagged HbMYB8-like protein (Figure 4b). In BiFC assay, *CgNLP1* was
343 translationally fused with the C-terminal portion of YFP (*CgNLP1*-cYFP), and
344 HbMYB8-like was fused with the N-terminal portion of YFP (HbMYB8-like -nYFP).

345 CgNLP1-cYFP and HbMYB8-like -nYFP were introduced into *A. tumefaciens* and
346 co-infiltrated into *N. benthamiana* leaves. Microscopic examination revealed YFP
347 fluorescence only when the two constructs were co-expressed. Leaves from plants
348 infiltrated with either of the constructs alone or in combination with the empty vector
349 showed no fluorescence (Figure 4c). These results demonstrated that CgNLP1
350 interacted with HbMYB8-like.

351 **HbMYB8-like protein localized on nucleus and induced necrosis**

352 HbMYB8-like-RFP fusion protein was transiently expressed in *N. benthamiana* by
353 agroinfiltration to determine the subcellular localization and explore the function on
354 defense response. In the leaf tissues expressing only RFP, red fluorescence was
355 observed throughout the entire cells. However, in the tissues expressing
356 HbMYB8-like-RFP fusion proteins, red fluorescence was observed only on the
357 nucleus (Figure 5a). After 2 days post infiltration with *A. tumefaciens* harboring
358 *HbMYB8-like-RFP* gene, significant necrosis was observed in the infiltration area of
359 tobacco leaf, while no necrosis was observed on the leaf infiltrated with *A.*
360 *tumefaciens* harboring only RFP gene (Figure 5b).

361 ***HbMYB8-like* responded to fungal phytopathogens and phytohormones in** 362 **rubber tree**

363 To explore the possible roles of *HbMYB8-like* in disease resistance of rubber tree, the
364 expression profiles of *HbMYB8-like* were investigated in responding to *C.*
365 *gloeosporioides* and *Erysiphe quercicola* which caused rubber tree anthracnose and
366 powdery mildew respectively. In the rubber tree leaves inoculated with *C.*
367 *gloeosporioides* and *E. quercicola*, the expression of *HbMYB8-like* was upregulated
368 significantly at 24 hr post inoculation (hpi) (Figure 6a-b). In addition, the expression
369 profiles of *HbMYB8-like* responding to different phytohormones were also
370 investigated. The results showed that the expression of *HbMYB8-like* was significantly
371 induced more than 8 times at 12 hours after SA treatment, and was significantly
372 down-regulated by JA and ET treatments (Figure 6c-e). These results suggested that
373 *HbMYB8-like* was involved in the resistance to fungal phytopathogens through SA,
374 JA and ET mediated signaling in rubber tree.

375 **CgNLP1 disrupted the nuclear accumulation of HbMYB8 and inhibited** 376 **HbMYB8-like induced cell death**

377 We had demonstrated that HbMYB8-like localized on the nucleus as a transcriptional
378 factor and induced necrosis in tobacco tissues (Figure 6). When we transiently
379 co-expressed CgNLP1-GFP fusion and nucleo-scytoplasmic marker (MIEL1-RFP) in
380 *N. benthamiana* leaves, the CgNLP1-GFP completely overlaps with the MIEL1-RFP,
381 indicating that CgNLP1 was localized on the nucleus and cell membrane. To
382 determine whether CgNLP1 interfered the subcellular localization of HbMYB8-like,
383 HbMYB8-like-RFP and CgNLP1-GFP were transiently co-expressed in *N.*
384 *benthamiana* leaves. Co-expression of HbMYB8-like-RFP with CgNLP1-GFP caused
385 a distinct change in the localization of HbMYB8-like-RFP in *N. benthamiana* cells.
386 By itself, HbMYB8-like-RFP was rather uniformly distributed in the nuclei. However,
387 in the presence of CgNLP1, less HbMYB8-like-RFP was observed in nuclei, and
388 instead a substantial amount of HbMYB8-like was localized on cell membrane.
389 CgNLP1-GFP was also detected on the nucleus and cell membrane (Figure 7a). In
390 addition, we observed that the size of necrosis caused by co-expression of
391 HbMYB8-like-RFP with CgNLP1-GFP was significant smaller than that caused by
392 expression of HbMYB8-like-RFP (Figure 7b and 7c). These data suggested that
393 CgNLP1 could interfere in the nuclear accumulation of HbMYB8-like to inhibit
394 HbMYB8-like induced necrosis.

395 **CgNLP1 inhibited SA signaling mediated by HbMYB8-like in tobacco leaves**

396 Since CgNLP1 could induce ethylene production in plants and HbMYB8 was
397 up-regulated in response to exogenous SA (Figure 2c and Figure 6), we examined the
398 expression patterns of genes related to SA signaling pathway in the tobacco leaves
399 expressing HbMYB8-like and co-expressing HbMYB8-like and CgNLP1. As showed
400 in Figure 8, the expression of *NtPR1a*, *NtPR1b*, *NtNPR1* and *NtPAL1* had been
401 enhanced significantly in the tobacco leaves expressing HbMYB8-like for 24 hours,
402 but reduced significantly in the tobacco leaves co-expressing HbMYB8-like and
403 CgNLP1. These results indicated that HbMYB8-like promoted SA signaling and
404 CgNLP1 repressed SA signaling mediated by HbMYB8-like.

405

406 **Discussion**

407 In the 30 years since the first NLP protein was discovered from the culture filtrate of
 408 *Fusarium oxysporum* f.sp. *erythroxyli* (Bailey, 1995), a large number of NLPs had
 409 been identified based on a prominent feature of the NPP1 domain with a conserved
 410 heptapeptide motif GHRHDWE (Oome & Van den Ackerveken, 2014). As mentioned
 411 in the introduction, NLP could be divided into three types, which differed especially
 412 in the number of cysteines. Type I NLPs contained two cysteines forming a conserved
 413 disulfide bond and type II had four cysteines forming two conserved disulfide bond and
 414 an additional putative calcium-binding domain (Oome et al., 2014). In our study,
 415 CgNLP1, an NLP protein identified from *C. gloeosporioides* vs. *hevea brasiliensis*,
 416 contained a NPP1 domain with two cysteine residues (Figure 1a), which conformed to
 417 the structural characteristics of type I NLPs. So, we identified it as type I NLP protein
 418 and it was also supported by phylogenetic analysis (Figure 1b). It had been suggested
 419 that signal peptide at N-terminal, two or four cysteine residues and heptapeptide motif
 420 GHRHDWE were directly related to the necrosis inducing ability of NLPs (Zaparoli
 421 et al., 2011; Lenarčič et al., 2017). When one cysteine was replaced, the NLP protein
 422 from *Phytophthora parasitica*, NPP1, lost necrosis inducing ability (Fellbrich et al.,
 423 2002). Both NLPPp and NLPPs, the NLPs from *P. parasitica* and *P. sojae* respectively,
 424 without signal peptide did not show cytotoxicity when transiently expressed in sugar
 425 beet (Qutob et al., 2006). In conserved heptapeptide motif GHRHDWE of NLP from
 426 *Moniliophthora perniciosa*, the substitution of either at His2 (H) or Asp4 (D) with
 427 alanine substantially abrogated its necrosis inducing activity (Ottmann et al., 2009;
 428 Zaparoli et al., 2011). However, HaNLP3, an NLP protein from *H. arabidopsidis*, did
 429 not induce necrosis on plant cells despite having a conserved heptapeptide motif
 430 (Ottmann et al., 2009; Cabral et al., 2012). In our case, CgNLP1 contained a signal
 431 peptide, two cysteine residues and an SHRHDWE motif with His2 (H) and Asp4 (D),
 432 despite which was different from the typical heptapeptide motif GHRHDWE at the first

433 amino acid site. However, CgNLP1 did not induce obvious necrosis and cell death
 434 when transiently expressed in tobacco leaves (Figure S3), suggested that signal
 435 peptide, the number of cysteine residue and the conserved SHRHDWE motif were not
 436 required for NLPs to induce necrosis and cell death. The mechanism of NLP induced
 437 necrosis requires more researches.

438 NLPs were suggested to play dual roles in plant pathogen interactions as virulence
 439 factors and as triggers of plant innate immune responses (Qutob et al., 2006). The
 440 deletion strains of NLP1 and NLP2 from *Verticillium dahlia* were found to be
 441 significantly less pathogenic on tomato plants (Santhanam et al., 2013). Gene
 442 silencing analysis showed that three NLPs from *P. capsica*, PcNLP2, PcNLP6, and
 443 PcNLP14, contributed positively to virulence on host plants (Feng et al., 2014).
 444 Introduction of Nep1 from *F. oxysporum* into a hypovirulent *Colletotrichum coccodes*
 445 strain dramatically increased its virulence and expanded its host spectrum (Amsellem
 446 et al., 2002). These data showed that some NLPs were required for virulence of
 447 phytopathogens. On the other hand, as described in the introduction part, some NLPs
 448 induced plant innate immune responses resulting in enhanced disease resistance (Seidl
 449 & Van den Ackerveken, 2019). For example, Ectopic expression of HaNLP3 in
 450 Arabidopsis enhanced the resistance to downy mildew (Oome et al., 2014). Our
 451 results showed that the disruption of *CgNLP1* impaired the pathogenicity of *C.*
 452 *gloeosporioides* to rubber tree, and ectopic expression of CgNLP1 in Arabidopsis
 453 enhanced the resistance to *Botrytis cinerea* and *Alternaria brassicicola* (Figures 3),
 454 indicating that *CgNLP1* performed dual functions as both virulence factor and elicitor.

455 Hormone signaling crosstalk played major roles in plant defense against pathogens
 456 (Derksena, et al., 2013). SA, JA and ET play critical roles in the regulation of
 457 signaling networks of basal resistance against multiple pathogens (Pieterse et al.,
 458 2012). SA signaling positively induces plant defense against biotrophic pathogens,
 459 whereas the JA/ET pathways are required for resistance predominantly against
 460 necrotrophic pathogens and herbivores insects (Yang et al., 2015). We had showed
 461 that CgNLP1 could enhance disease resistance of Arabidopsis to necrotrophic fungi *B.*
 462 *cinerea* and *A. brassicicola* (Figure 3), and had also detected that CgNLP1 promoted

463 ethylene synthesis and accumulation in plant tissues (Figure 2c), indicating that the
464 disease resistance triggered by CgNLP1 to necrotrophic pathogens may be related to
465 ethylene accumulation and enhanced ethylene mediated signaling pathway.

466 Fungal effectors were usually secreted and delivered into host plants as pathogenic
467 factors to shield the fungus, suppress the host immune response, or manipulate host
468 cell physiology through targeting plant defense components, signaling, and metabolic
469 pathways to promote host plant colonization (Lo Presti et al., 2015). Plant
470 transcription factors (TFs) played roles in diverse biological processes including
471 defense responses to pathogens (Seo & Choi, 2015). Thus, plant transcription factors
472 are logical targets for effectors, and several studies had demonstrated this. A bacterial
473 effector AvrRps4 interacted with WRKY transcription factors (Sarris et al., 2015). A
474 *Phytophthora infestans* effector Pi03192 prevented the translocation of NAC
475 transcription factors from the endoplasmic reticulum to the nucleus (Hazel et al.,
476 2013). PpEC23 from *Phakopsora pachyrhizi* targeted transcription factor GmSPL121
477 to suppress host defense response (Qi et al., 2016). Stripe rust effector PstGSRE1
478 disrupts nuclear localization of transcription factor TaLOL2 to defeat ROS-induced
479 defense in wheat (Qi et al., 2019). In our study, CgNLP1 physically interacted with
480 HbMYB8-like, a R2R3 type MYB transcription factor in rubber tree, which was
481 localized to the nucleus (Figure 5a). The expression of CgNLP1 in tobacco leaves
482 induced partial re-localization of HbMYB8-like from nucleus to plasma membrane
483 and reduced the amount of HbMYB8-like in nuclei (Figure 7a). Logically, it was not
484 necessary for CgNLP1 to make the long journey from the nucleus to bring
485 HbMYB8-like back to the plasma membrane if only to inhibit the function of
486 HbMYB8-like, so it remained to determine the physiological function of
487 HbMYB8-like re-localization.

488 SA was believed to play important roles in the regulation of programmed cell death or
489 hypersensitive reaction in plants, a form of plant defense (Radojičić et al., 2018).
490 According to our data, CgNLP1 contributed to the virulence of *C. gloeosporioides* on
491 rubber tree (Figure 2a and 2b), and its target in rubber tree, HbMYB8-like, induced
492 typic necrosis and cell death (Figure 5b), moreover, CgNLP1 repressed cell death

induced by HbMYB8-like in tobacco leaves (Figure 7b and 7c). In addition, it also showed that HbMYB8-like enhanced the expression of genes related to SA signaling pathway such as *NtPR1a*, *NtPR1b*, *NtNPR1* and *NtPAL1*, which were inhibited significantly when CgNPL1 co-expressed with HbMYB8-like (Figure 8). Consider the above data, it looked like that the pathogenicity of CgNLP1 was achieved through inhibition of defense induced by HbMYB8-like mediated SA signaling.

In summary, we identified an important *C. gloeosporioides* effector CgNLP1 which played dual roles as virulence factor to rubber tree and as a trigger of plant defense responses, and CgNLP1 blocked nuclear accumulation of rubber tree transcription factor HbMYB8-like and repressed SA signaling mediated by HbMYB8-like. This extended our knowledge of novel pathogenic strategy mediated by effector CgNLP1 for *C. gloeosporioides* on rubber tree.

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Figure legends

Fig. 1 Multiple sequence alignment and Phylogenetic analysis of CgNLP1. (a) Alignment of the NLP proteins from different microorganism. Shading indicated regions of conservation in all (black), the same amino acid as CgNLP1 (grey) of sequences. NLPs used for alignment are from *Fusarium oxysporum* (AAC97382.1), *Pythium aphanidermatum* (AAD53944.1), *Phytophthora infestans* (AAK25828.1), *Phytophthora parasitica* (AAK19753.1), *Phytophthora sojae* (AAK01636.1), *Streptomyces coelicolor* A3(2) (CAB92890.1), *Alkalihalobacillus halodurans* C-125 (BAB04114.1) and *Vibrio pommerensis* (CAC40975.1). (b) Phylogenetic tree of CgNLP1 with different type of NLPs in fungi. VdNLP1 and VdNLP3 are from *Verticillium dahlia*, DserNEP3 is from *Diplodia seriata*, FoNEP-like is from *Fusarium oxysporum*, BcNEP1 and BcNEP2 are from *Botrytis cinerea*, DserNEP2 is from *Diplodia seriata*, PcNPP1 is from *Phytophthora cinnamomi*, MoNLP2 and MoNLP3 are from *Pyricularia oryzae*, VdNLP4, VdNLP7, VdNLP8 and VdNLP9 are from *Verticillium dahlia*.

Fig. 2 CgNLP1 contributed to the virulence of *C. gloeosporioides* to rubber tree and induced ethylene production in plants. (a) Disease Symptoms on rubber tree leaves at 4 days post inoculated with conidia of Δ CgNLP1, Res- Δ CgNLP1 and WT. (b) Statistic analysis of lesion diameter after inoculation with WT, Δ CgNLP1 and Res- Δ CgNLP1. (c) The ethylene content in tobacco leaves expressing CgNLP1. pEGAD represented the tobacco leaves expressing empty vector pEGAD and pEGAD-CgNLP1 represented the tobacco leaves expressing constructive vector pEGAD-CgNLP1. (d) The expression pattern of *NtACO1* (LOC107781126) in tobacco leaves expressing CgNLP1. (e) The expression pattern of *NtACSI* (LOC107831434) in tobacco leaves expressing CgNLP1 at different time points. Data are shown as the means $\pm$ SD from three independent experiments and columns with different letters indicate significant

700 difference ($P < 0.05$).

701 **Fig. 3** Overexpression of *CgNLP1* in Arabidopsis enhanced the resistance to *B.*
702 *cinerea* and *A. brassicicola*. (a) Disease Symptoms on Arabidopsis lines
703 overexpressing *CgNLP1* (*CgNLP1*-OE) and wild type (Col-0) at 3 days post
704 inoculated with *B. cinerea* and *A. brassicicola*, respectively. (b) Statistic analysis of
705 lesion diameter on *CgNLP1*-OE and *Col-0* after inoculation with *B. cinerea* and *A.*
706 *brassicicola*. Data are shown as the means $\pm$ SD from three independent experiments
707 and columns with different letters indicate significant difference ($P < 0.05$).

708 **Fig. 4** Screening and verification of the interaction between *CgNLP1* and
709 HbMYB8-like. (a) Verification of the interaction between *CgNLP1* and
710 HbMYB8-like by yeast two-hybrid assay. AD indicates pGADT7, BD indicates
711 pGBKT7, pGADT7-T + pGBKT7-53 indicates a positive control, and
712 pGBKT7-Lam+pGADT7-T indicates the negative control. SD/-Leu/-Trp/X- α -gal
713 indicates the medium with X- α -gal, but lacking Leu and Trp.
714 SD/-Ade/-His/-Leu/-Trp/Xa-gal/Aba indicates the medium with X- α -gal and Aba, but
715 lacking Ade, His, Leu and Trp. 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} respectively refer to
716 the yeast suspension with the initial OD value of 2.0 being diluted in a 10-fold
717 gradient. (b) Verification of the interaction between *CgNLP1* and HbMYB8-like by
718 GST pull-down assay. His and GST stand for pColdTM TF and pGEX respectively.
719 Arrows indicate GST- and His-tagged proteins. (c) Verification of the interaction
720 between *CgNLP1* and HbMYB8-like by bimolecular fluorescence complementation
721 (BiFC) assay. cYFP+nYFP, *CgNLP1*-cYFP+nYFP, cYFP+*HbMYB8-like*-nYFP were
722 used as a negative control. Scale bar $\square = \square 25\mu\text{m}$.

723 **Fig. 5** HbMYB8-like localized on nucleus and induced necrotic cell death. (a)
724 Subcellular localization of HbMYB8-like protein in tobacco leaves. (b) Cellular
725 necrosis induced by HbMYB8-like in tobacco leaves. Leaves were photographed
726 under UV illumination (right) and normal light (left).

727 **Fig. 6** Expression profiles of *HbMYB8-like* in rubber tree the leaves with
728 phathomycete inoculation and phytohormones treatments. Data are shown as the
729 means $\pm$ SD from three independent experiments and columns with different letters

730 indicate significant difference ($P < 0.05$).

731 **Fig.7** CgNLP1 repressed nuclear accumulation of HbMYB8-like and inhibited
732 necrosis induced by HbMYB8-like. (a) CgNLP1 repressed nuclear accumulation of
733 HbMYB8-like. Scale bar $\square = \square 25\mu\text{m}$. (b) CgNLP1 inhibited necrosis induced by
734 HbMYB8-like. (c) Statistical analysis of necrosis diameter in tobacco leaves. Data are
735 shown as the means $\pm$ SD from three independent experiments and columns with
736 different letters indicate significant difference ($P < 0.05$).

737 **Fig. 8** Expression patterns of genes related to SA signaling pathway in the tobacco
738 leaves expressing HbMYB8-like and co-expressing HbMYB8-like and CgNLP1. Data
739 are shown as the means $\pm$ SD from three independent experiments.

740

741 **Supporting Information**

742 Additional supporting information may be found in the online version of this article.

743 **Fig. S1** Nucleotide sequence and deduced amino acid sequence of *CgNLP1*. Shading
744 indicates the amino acid sequences of signal peptide.

745 **Fig. S2** Generation and molecular confirmation of *CgNLP1* deletion mutant
746 (ΔCgNLP1) and complementation mutant (Res- ΔCgNLP1). (a) The diagram of
747 *CgNLP1* knockout vector. (b) Diagnostic PCR analysis for deletion of *CgNLP1* and
748 integration of *CgNLP1* into the genome of *C. gloeosporioides*. (c) The expression
749 level of *CgNLP1* in WT, ΔCgNLP1 and Res- ΔCgNLP1 by quantitative RT-PCR.

750 **Fig. S3** Trypan blue transient of tobacco leaves expressing *CgNLP1*. pEGAD
751 indicates the tissue expressing empty vector pEGAD-eGFP, pEGAD-CgNLP1
752 indicates the tissue expressing recombinant vector pEGAD-CgNLP1-eGFP.

753 **Fig. S4** Identification of *CgNLP1* transgenic Arabidopsis lines by Western blot. Col-0
754 indicated Arabidopsis Columbia-0 and 1-6 indicated different CgNLP1-OE transgenic
755 lines under Col-0 background.

756 **Fig. S5** Alignment of HbMYB8-like and homologs from different plants. Shading
757 indicated regions of conservation in all (black), the same amino acid as HbMYB8-like
758 (grey) of sequences. The protein sequences used for alignment are: *Arabidopsis*
759 *thaliana* AtMYB6 (EFH48703.1), *Arabidopsis thaliana* AtMYB8 (Q9SDS8.1), *Oryza*

760 *sativa* OsMYB30 (Q6K1S6.1), *Gossypium hirsutum* GhMYB1 (NP_001313761.1),
 761 *Triticum aestivum* TaRIM1 (AMP18876.1).

762 **Fig S6** Phylogenetic tree of HbMYB8-like with different types of MYB proteins in
 763 plants.

764 **Table S1** The primers used for PCR amplification quantitative and RT-PCR.

* 20 40 60 80 100
CgNLP1 : --MRSDVLTIPALASGFSAS--RSNLGRGEGVHDEISESSEKQ-VQDN*VPGKAIEREN*PLHIAHGCCOPYTAVNDAGDTSGLLKPTGSSSTGGCK-DTS : 95
 AAK97382.1 : MHFQTFINALVIALPATGMAASESGLINLIRHAAVYNHDSISENVKKT-IEGGAISAI*EDWOLHLHIDGAGASPTAYDTNAGVSSGGLQDSGSKTGCK-DTS : 98
 AAK19753.1 : ----MNVLTITLRAAVSLAV-----VQADVISHDAVVPKPP-TATTTEQKAGVKRKEQIHISNGGHP*PAPVDAMGNTSSGLKPTGSSSACGSGSY : 87
 AAK25828.1 : ----MNIQLFASAAALTY-----AHADVISHDAVIEPT*TTTTEQKAGIKRKEQIHISNGGHP*PAVDADGNTSSGLNPTGSSSACGSGSY : 87
 AAK01636.1 : ----MNLRPATLTLISFAY-----VSASVINHDGVVPTTP-TPTTALQAVKYKREQIHISNGGHP*PAPVDNNGNTSSGLNPTGSSSACGSGSY : 88
 RAD53944.1 : ---MVRFVSALLTPAAGVLAS-----TNAAVINHDVAVVWPP-P-EPADATQALAVYKREQIDVVGCCOPYPAVDPGNGTSSGLKPTGSSQAACAK-IMS : 88
 BAB04114.1 : MMRVFVIGLLSILASISLVSE-----ANAADVHDKVVGVFEV-TPTTITQVAKRQOYITVSTGSGVPPVADNGTSSGLNPTGPHNGGCS--SH : 90
 CAB92890.1 : ---MSRPPTTPPRRRLLTVT-----LGALALVAFAPATAAAPPGLPGNADGLERYMQAPDYDDTDCGYPTPT*IGADGAVNGSLNPTGALNENORDASD : 92
 CAC40975.1 : ----MKKGLYTL*LLNTTILE-----VSESVFTHLEQALPTKVINIGQ--PMDFDNDSDCYPSAGISKYQQNRGLNVTGGITTCRRARDF : 80

* 120 140 160 180 200
CgNLP1 : KGQT--YARGTTLNG--KPAIMYANYFKPDMDENQGVFVSGSHRHDWES*DWLVN---NTPSPAI*VGGASGHGDFRLNNPQR--GDGSKVKEYFT : 183
 AAK97382.1 : KGQT--YARAAMHNG--KPAIMYANYWPKDMDENQGVFVSGSHRHDWEVNVVFDIN--YQSPGATLYAAASGHGDKTKTKNQR--SGNNVMAEYFT : 186
 AAK19753.1 : GSDV--YGRVALYNG--VVAIMYSYVYFKPDSVET--LGRHRHDWEHVVVWVDD--IKLDS*SI*IAVSPSAHSGYNIYYPESNTIDGYSAKVWYSS : 175
 AAK25828.1 : GSDI--YGRVALYNG--VVAIMYSYVYFKPDSLTF--LGRHRHDWEHVVVWVDD--IKLDS*SVIAVSPSAHSGYNIYHPESENTIDGYSTKWYSS : 176
 AAK01636.1 : GTOI--YGRVAVYQG--VVAIMYSYVYMKPDKETLT--LGRHRHDWCAVWVWVDD--TAASRP*VALSASASGYNKYHPESSSYFSGNARIDYSS : 175
 RAD53944.1 : KAVV--YSSSGSYNG--VVAIMYSYVYMKPDKSEPT--LGRHRHDWEHVVVWVDN--IASAN--I*VALSASASGYKRFPAADKSYLDGIFATYSKS : 177
 BAB04114.1 : IGCV--YSSSGWHNG--VVAIMYSYVYFKPDS*SP--LGRHRHDWEHVVVWVDN--PANPS*QLLSIYSQHNQYVNPVATDNTRE*QFPLRUSH : 174
 CAB92890.1 : LDNNGHRAKCDGQ---WCHYHGLDYSKDKDALPETSLSGRHRHDWEHVVVVREGTVGYVSTSNHGSFVSHERSAVRFDGTHPKVIVYHK*GIGTHCR : 188
 CAC40975.1 : LSSSNTLHRYAC*DSQGMYSYCGHPSYVYFKPDQVTAYRDLFSGSHRHDWEHVAVITKNGVITHASYS*HGLKLNKTPITQTAREGDHVKFVYHK*GVGTHAHR : 180

GRRHDWE

(a)

(b)

(c)

(d)

(e)

(a)

B. cinerea

A. brassicicola

(b)

B. cinerea inoculation

(c)

A. brassicicola inoculation

(a)

(b)

(c)

(a)

RFP

DAPI

Bright

Merged

(b)

Bright

UV

(a)

(b)

(c)

(d)

(e)

(a) **(b)** **(c)**

(a)

NtNPR1

(b)

NtPR1a

(c)

NtPR1b

(d)

NtPAL