Arabidopsis *PEN2*, a promising gene in upraising penetration resistance against rice necrotrophic fungus *Rhizoctonia solani*

Daraksha Parween [1] , **Eram Sultan** [1] , **Kalpana Dalei** [1] , **Binod Bihari Sahu** [Corresp. 1]

1 Life Science, National Institute of Technology Rourkela, Rourkela, Odisha, India

Corresponding Author: Binod Bihari Sahu
Email address: binodbiharisahu@gmail.com

Rhizoctonia solani, a soilborne necrotroph, causes sheath blight in rice which poses a major threat to global rice production. Besides rice sheath blight, it has a wide host range of other economically important crops like soybean, sugarcane, maize etc. Despite being the most hostile fungus, the mechanism involved in the *R. solani* pathobiology is poorly understood. Non-host resistance (NHR) is an emerging concept that allows breeders to transfer traits to food crops that would impart a broad-spectrum disease resistance. Several NHR genes are known to function against different pathogens of which Arabidopsis *PEN1, PEN2* and *PEN3* have been reported to limit the entry of non-adapted powdery mildews and provide cell wall based defenses against different fungi. Till now, there has been no study regarding the involvement of these *PEN* genes against *R. solani.* In this study, we have screened *pen1, pen2-3* and *pen3-1* against *R. solani* to explore their contribution in penetration resistance. Among the three *pen* mutants studied, *pen2-3* allowed maximum penetration during the early hours of infection. *R. solani* colonization was also observed in *pen1* and *pen3-1* but the effect was less drastic than *pen2-3*, suggesting the involvement of *PEN2* in pre-invasive defense. To validate our hypothesis, we screened a complemented *pen2* accession, *PEN2-GFP*, which showed restored penetration resistance comparable to Col-0. Altogether, our results demonstrate that *PEN2* is involved in pre-penetration resistance, and contributes to NHR by enhanced disease resistance to *R. solani.*

1   **Arabidopsis *PEN2*, a promising gene in upraising penetration resistance against rice**

2   **necrotrophic fungus *Rhizoctonia solani***

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4   Daraksha Parween, Eram Sultan, Kalpana Dalei and Binod Bihari Sahu*

5   Laboratory of Molecular Genetics and Plant Immunity, Department of Life Science, National

6   Institute of Technology Rourkela, Odisha, INDIA, 769008

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8   *To whom correspondence may be addressed. E mail: binodbiharisahu@gmail.com

9

10   **Email Addresses:**

11   Daraksha Parween: parweendaraksha8@gmail.com

12   Eram Sultan: sultan.eram@gmail.com

13   Kalpana Dalei: kalpanadalei2034@gmail.com

14   Binod Bihari Sahu: binodbiharisahu@gmail.com

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Abstract

Rhizoctonia solani, a soilborne necrotroph, causes sheath blight in rice which poses a major threat to global rice production. Besides rice sheath blight, it has a wide host range of other economically important crops like soybean, sugarcane, maize etc. Despite being the most hostile fungus, the mechanism involved in the *R. solani* pathobiology is poorly understood. Non-host resistance (NHR) is an emerging concept that allows breeders to transfer traits to food crops that would impart a broad-spectrum disease resistance. Several NHR genes are known to function against different pathogens of which Arabidopsis *PEN1, PEN2* and *PEN3* have been reported to limit the entry of non-adapted powdery mildews and provide cell wall based defenses against different fungi. Till now, there has been no study regarding the involvement of these *PEN* genes against *R. solani*. In this study, we have screened *pen1, pen2-3* and *pen3-1* against *R. solani* to explore their contribution in penetration resistance. Among the three *pen* mutants studied, *pen2-3* allowed maximum penetration during the early hours of infection. *R. solani* colonization was also observed in *pen1* and *pen3-1* but the effect was less drastic than *pen2-3*, suggesting the involvement of *PEN2* in pre-invasive defense. To validate our hypothesis, we screened a complemented *pen2* accession, *PEN2-GFP*, which showed restored penetration resistance comparable to Col-0. Altogether, our results demonstrate that *PEN2* is involved in pre-penetration resistance, and contributes to NHR by enhanced disease resistance to *R. solani*.

Key Words: Nonhost resistance; necrotroph; *Rhizoctonia solani*; infection cushion; penetration

Introduction

Rhizoctonia solani (teleomorph, *Thanatephorus cucumeris*), a multinucleated filamentous necrotroph, causes diseases like sheath blight and banded leaf disease in monocots like rice,

40   maize and sorghum; aerial blight and stem rot in legumes like mung bean and soybean; sheath

41   rot in sugarcane, damping off of cotton, black scurf and sprout canker in potato, heart rot in

42   cabbage, and foliar blights in other fruits and plantation crops (Ajayi-Oyetunde & Bradley 2018;

43   Nagaraj et al. 2017). Rice sheath blight is the most devastating disease challenging global food

44   security amongst other diseases caused by *R. solani* and can potentially cause around 50%

45   reduction in rice yield worldwide (Zheng et al. 2013). *R. solani* is both soilborne as well as

46   waterborne pathogen. This non-sporulating fungus survives in the form of sclerotia during the

47   inactive phases of its infection cycle (KUMAR[1] et al. 2009). Under favorable conditions, these

48   sclerotias germinate into mycelia which further form atypical hyphal aggregates called infection

49   cushions, required for host penetration (Kumar et al. 2011; Łaźniewska et al. 2012; Singh et al.

50   2012; Taheri & Tarighi 2011). *R. solani* induces programmed cell death with loss of

51   photosynthetic activity and development of necrotic lesions in the host tissues (Taheri & Tarighi

52   2011) (Mondal et al. 2012). It is also reported that the hyphal growth and penetration of *R. solani*

53   is influenced by protrusions or openings on the leaves of host surface, such as trichomes, stomata

54   or papillae (Basu et al. 2016).

55   Most of the plant diseases are caused by fungal pathogens (Łaźniewska et al. 2012). To fight

56   against various pathogenic attacks, plant possess immune system with multilayered continuum of

57   both pre-formed and acquired barriers. The disease resistance is mediated by sequential basal and

58   resistance (R)-gene mediated hypersensitive response that does not always involve recognition of

59   pathogenic cues (Gill et al. 2015). Non-host resistance demarcates the host range of

60   phytopathogenic microorganisms, representing the hallmarks of basic compatibility. Thus,

61   adapted pathogens always try to suppress or evade the plant's basal defense mechanism by

62   secreting a repertoire of effector molecules (Speth et al. 2007) against robust and durable nature

63   of NHR as a part of innate immunity (Nuernberger & Lipka 2005). Durability of NHR has

64   increased attention to revamp resistance in crop. Elaborated suite of plant defense system induces

65   downstream cell-autonomous responses of PAMP-triggered immunity (PTI) including

66   production of reactive oxygen species (ROS), MAP-kinase signaling, transcriptional induction of

67   pathogenesis-related (PR) genes, and callose deposition (Bittel & Robatzek 2007).

68   In Arabidopsis, cell wall based defenses are mediated by three *PENETRATION* genes - *PEN1,*

69   *PEN2* and *PEN3* and allow limited entry of non-adapted powdery mildews. *PEN1* encodes a

70   syntaxin (SYP121/PEN1) which belongs to the SNARE superfamily proteins. *PEN1* plays a role

71   in the papilla formation (Collins et al. 2003). *PEN2* encodes myrosinase, associated with

72   peroxisomes, which implicates production of glucosinolate derivatives as an antifungal defense

73   compounds (Bednarek et al. 2009; Lipka et al. 2005). The toxic by-products of PEN2 are

74   transported to the sites of pathogen entry by ABC transporter proteins which is encoded by

75   *PEN3* gene. *PEN2* and *PEN3* have been documented to confer disease resistance against

76   biotroph *viz. Erysiphe pisi,* hemibiotrophic oomycete *Phytophtora infestans* as well as

77   necrotroph *P. cucumerina* (Stein et al. 2006).

78   The molecular mechanism of disease resistance to *R. solani* and its mode of infection in hosts are

79   not clear. Therefore, the objective of this study was to unravel the defense strategies against *R.*

80   *solani* in non-host wild type Arabidopsis Col-0 and *pen1, pen2-3* and *pen3-1* mutants.

81

Materials and Methods

Growth and maintenance of Arabidopsis

Arabidopsis wild type; Col-0 (N1093), T-DNA insertion line of *pen1* (N673657,

SALK_004484C), EMS mutants *pen2-3* (N66946) and *pen3-1* (N66467) and complement *PEN2-*

GFP (N67162) were procured from NASC, Europe and were grown in the plant growth chamber.

The plants were grown on soil mixture containing agropeat: vermiculite (3:1), and maintained at

14hour photoperiod with ~100 $\mu E/m^2/s$ light intensity, 21°C temperature and 60% humidity.

Rhizoctonia solani culture conditions

R. solani isolate was collected from National Rice Research Institute (NRRI) and was routinely

cultured on freshly prepared potato dextrose agar (PDA) medium supple

mented with antibiotic streptomycin (100 μg/mL). PDA plates were grown for ~14 days until

sclerotias developed which were used for infection assay.

Infection assay

Detached leaf assay was performed by taking three upper rosette leaves of 4 week old plants of

Arabidopsis (Mukherjee et al. 2010). Leaves were inoculated with approximately equal sized

(~0.3-0.4 cm diameter) sclerotia of *R. solani* and maintained in petriplates with 100% humidity.

Infected leaves were harvested at different time points for microscopic and macroscopic

observations. The experiment was carried out three times, and each contained three biological

replicates.

Microscopy

Trypan blue, DAB (3,3'-diaminobenzidine) and aniline blue staining were performed to study

cell death, H_2O_2 accumulation and callose deposition respectively, as described by Park et al

(2009) (Park et al. 2009). For trypan blue staining, infected leaves were cleared in alcoholic

lactophenol (2:1) and stained with 250 μg/mL trypan blue in lactophenol (phenol: glycerol: lactic

106   acid: water – 1:1:1:1, v/v). It was further destained with lactophenol, mounted onto glass slide

107   with 50% glycerol and examined under bright field microscope. For DAB staining, infected

108   leaves were incubated in 1mg/mL aqueous DAB solution for 8h in dark following which stain

109   was replaced with water and incubated in similar conditions. Destaining was performed with

110   acetic acid: ethanol (96:4 v/v) and mounted with 50% glycerol and visualized under brightfield

111   microscope. For aniline blue staining, leaves were cleared in alcoholic lactophenol and stained

112   overnight with 0.01% aniline blue in 150mM K_2HPO_4, pH 9.5. stained leaves were equilibrated

113   with 50% glycerol and observed under UV excitation.

114   ### Extraction and Estimation of Chlorophyll content

115   One gram of treated and untreated leaves were taken at 30 and 48hpi and ground with 20 mL of

116   80% acetone. It was then centrifuged at 5000 rpm for 5 minutes. The supernatant was collected

117   in a tube and the process was repeated until the residue appears colorless. Supernatant was made

118   upto 100 mL with 80% acetone. The absorbance of the solution was recorded at 645 nm and 663

119   nm against the blank solvent (acetone) (Rajalakshmi & Banu 2013). The concentrations of

120   chlorophyll a, chlorophyll b and total chlorophyll was measured as mg/g of the sample and were

121   calculated as described by Rafii et al. 2015 (Azizi et al. 2015) using the following equation:

122

123   Chlorophyll a (mg/g Fresh leaf) = $12.7 \times (A663) - 2.69\left(\frac{A645}{1000}\right) \times \frac{V}{W}$

124

125   Chlorophyll b (mg/g Fresh leaf) = $22.9 \times (A645) - 4.68\left(\frac{A663}{1000}\right) \times \frac{V}{W}$

126

127   Total Chlorophyll (mg/g Fresh leaf) = $20.2 \times (A645) + 8.02\left(\frac{A663}{1000}\right) \times \frac{V}{W}$

128 ### Scanning electron microscopy

129 Infected leaves were fixed in 4% paraformaldehyde and subsequently washed with phosphate

130 buffer. Leaves were blotted dry with kimwipe and mounted on metal stubs for visualization

131 under environmental SEM. Each plant had a number of three detached leaves and data from three

132 biological replicates.

133 ### Pathogenicity Assays and calculation of disease level using detached leaves

134 The infected rosette leaves from the wild type and *pen* mutants, harvested at 1dpi, 2dpi and 3dpi

135 were photographed. The percentage of disease level was calculated based on the area of necrotic

136 lesions formed at different time point in all accessions of Arabidopsis. At 1dpi, 2dpi and 3dpi,

137 disease level was calculated in whole plant as follows:

138

139 $$\text{Percentage of disease level (\%)} = \left(\frac{Area\ of\ lesion\ formed\ in\ leaves\ of\ a\ plant}{total\ area\ of\ leaves\ of\ a\ plant} \right) \times 100$$

140

141 Each plant had a number of three detached leaves and data from three biological replicates were

142 taken for calculation. The data were expressed as means ± standard error of the mean (SEM).

143

144 # Results

145 ### *PEN2* is involved in pre-penetration resistance to *R. solani*

146 In order to assess the resistance phenotypes in Arabidopsis wild type Col-0 and penetration

147 deficient mutants, a T-DNA insertion mutant *pen1* and EMS generated *pen2-3* and *pen3-1* were

148 used. The *pen* mutants obtained from NASC were confirmed for their homozygosity by using

149 PCR based analysis (Fig. S1, Supplementary Table 1). Leaves of four weeks old plants were

150 challenged with *R. solani* sclerotia for 6, 12, 24, 30, 36 and 48 hours post inoculation (hpi). We

151   observed no mycelial growth after 6 and 12hpi in either Col-0 or the *pen* mutants. However, at

152   24hpi and 30hpi, onset of branching of secondary hyphae were observed in Col-0 but no such

153   extensive mycelial colonization was observed (Fig. S2). On the other hand, *pen* mutants showed

154   profuse branching of the fungal hyphae. Among the *pen* mutants, *pen2-3* showed maximum

155   fungal colonization and initiation of typical infection cushions was observed at 30hpi, which

156   lacked in wild type as well as *pen1* and *pen3-1*. Prominent infection cushions were clearly visible

157   at 36hpi and 48hpi in *pen2-3*, which was initiated in *pen3-1* at 48hpi and totally absent in Col-0

158   and *pen1* (Fig. S2). To further examine the results obtained in previous time course experiment,

159   we focused our observations on 30hpi where initiation of typical infection cushion was seen in

160   *pen2-3* and 48hpi where well-formed infection cushions were observed (Fig. 1). Leaves of *pen2-*

161   *3* showed several runner hyphae entangled together to form lobate appressoria and initiation of

162   infection cushion at 30hpi. At 48hpi, there was more hyphal proliferation in *pen2-3* which

163   resulted in formation of compact bundles of fungal hyphae entrenched onto the leaves surface

164   and the formation of infection cushions (Fig. 1).

165   *R. solani* infection reduces photosynthetic efficiency in *pen2-3*

166   Pathogen virulence leads to the inhibition of photosynthesis (Okorski et al. 2008). Thus, the

167   disappearance of chlorophyll was analyzed at 30 and 48hpi. Compared to the untreated leaves of

168   Arabidopsis, treated leaves showed reduced chlorophyll content. Higher amount of chlorophyll

169   content was recorded in treated leaves of Col-0 with that of *pen* mutants. *pen2-3* showed

170   maximum degradation in cellular chlorophyll content as compared to wild type, *pen1* and *pen3-1*

171   which correlate with the microscopic observations (Fig. S3).

pen2-3 triggers the formation of infection cushions

We investigated the stages of formation of typical infection cushion of *R. solani* on leaves by comparing wild type and *pen* mutants by observing under SEM. Col-0 showed secondary hyphal branching at 30hpi and no infection cushions *pen1* showed almost similar hyphal structure as that of Col-0 (Fig. S4). However, *pen2-3* at 30hpi, showed profuse mycelial branching and initiation of infection cushions (Fig. S4) which however was observed in *pen3-1* at 48hpi. At 48hpi, *pen2-3* formed infection cushions which leads the hyphae to a bulbous end forming lobate appressoria (la). Aggregation of compact hyphal branches leads to the formation of infection pegs at the base of the cushion which allows the pathogen to penetrate and proliferate further (Dodman et al. 1968). The growth of the hyphae on the leaf surface of Arabidopsis showed usual right-angled branching patter of *R. solani.* The infection cushions were found to be present exclusively in *pen2-3.*

pen mutants accumulate more H_2O_2 and callose in response to *R. solani* infection

Production of ROS and deposition of callose by plants acts as a major defense response against biotic stress at the site of plant-pathogen interaction (Fauth et al. 1998; Luna et al. 2011). Deposition of H_2O_2 was detected as dark yellowish-brown precipitate (Fig. 2). As expected, *pen2-3* exhibited relatively higher level of accumulation of H_2O_2 at more sites of infection, followed by *pen1* and *pen3*-1 as compared to Col-which can be correlated with increased cell death rate (Fig. 2). Callose deposition was examined using aniline blue stain. The leaves of wild type showed reduced callose deposition and mutant plants accumulated significantly more localized callose (Fig. 3). Significantly higher amount of callose deposition was found in the leaves of infected *pen2-3* among all indicating compromised disease resistance in *pen2-3,* substantiating the microscopic data.

195   Macroscopic lesion phenotypes induced by *R. solani* vary between wild type and *pen*
196   mutants of Arabidopsis

197   Wild type Col-0 and the *pen* mutants were almost indistinguishable up to 1dpi (days of post

198   inoculation) with sclerotia of *R. solani.* The wild type became chlorotic upon increasing the

199   inoculation time up to 3dpi and did not develop much lesions spontaneously. In contrast to that

200   *pen1*, *pen2-3* and *pen3-1* started showing necrotic lesions along with chlorosis at 2dpi. *pen2-3*

201   showed maximum necrotic lesion and chlorosis at 2dpi and 3dpi. Macroscopic lesions were

202   almost identical up to 1dpi. By contrast, when the infection period was increased up to 3dpi,

203   mutants were clearly distinguished from the wild type, especially as seen in leaves of *pen2-3*

204   mutant (Fig. 4). Based on this experiment, percentage of disease severity was calculated (Fig.

205   S5). Wild type Col-0 had an average of 0.09%, 0.55%, 22.08% necrotic tissues at 1dpi, 2dpi and

206   3dpi respectively (average ± standard deviation, n= 5). *pen1* showed an average necrotic lesion

207   of about 0.102%, 0.064%, 27.06% at 1dpi, 2dpi and 3dpi respectively. On the other hand, *pen2-3*

208   mutant leaves showed maximum necrotic lesion with an average of 0.139%, 36.38% and 67.54%

209   at 1dpi, 2dpi and 3dpi respectively. Leaves of *pen3-1* showed relatively lesser necrotic lesions

210   than *pen2-3* but more than *pen1* and Col-0 with an average of 0.108%, 2.71% and 43.54% at

211   1dpi, 2dpi and 3dpi respectively.

212   *PEN2* provides penetration resistance against *R. solani*
213   To authenticate the result that *pen2-3* mutant triggers cushion formation, we targeted

214   complement line expressing green fluorescent protein (GFP)-PEN2, driven by native 5'

215   regulatory region in PEN2-1 background. Due to the knockdown of *PEN2*, *pen2-3* allowed the

216   formation of infection cushions on the leaves surface. Furthermore, the complemented line

217   (PEN2-GFP) for the *pen2-3* mutant, behaved similar to wild type Col-0 with no cushion

218   formation at 30hpi and 40hpi in response to *R. solani* (Fig. 5). This experiment corroborated our

219   hypothesis that *PEN2* enhance penetration resistance against *R. solani.*

Discussion

221   For a successful plant- pathogen interaction, the pathogen recognizes a surface for the initiation

222   of its growth and further penetrates into the host tissue to cause infection. Non-host resistance

223   provides durable protection against invading pathogens by using various defense strategies

224   (Thordal-Christensen 2003). The coherent investigation of our present study revealed that *pen2-3*

225   contributes to non-host resistance in *Arabidopsis thaliana* to *Rhizoctonia solani,* a necrotrophic

226   rice pathogen. Among the *pen* mutants, including *pen1, pen2-3 and pen3-1*, *pen2-3* was observed

227   with compromised penetration resistance to *R. solani*. *pen2-3* showed maximum number of

228   atypical infection cushions which clearly depicts the important role playing in non-host

229   resistance against sheath blight in rice.

230   Flentje (1963) reported that the infection cushions develops only in the susceptible host which is

231   suppressed in the resistant host (Flentje et al. 1963). Despite having very less in-depth studies

232   about the mechanism of infection process of *R. solani* in various host, it is reported that *R. solani*

233   produces typical infection structure by forming cluster of hyphae with bulbous end during its

234   pre-penetration stage(Łaźniewska et al. 2012). This clustering T-shaped branched hyphae and

235   formation of infection cushions further leads to penetration by defeating the barriers present in

236   the host tissue, enters via penetration peg and promotes colonization (Pannecoucque & Höfte

237   2009). To fight against the invasion of pathogens, Arabidopsis have penetration genes (*PEN*)

238   which avoid penetration and is responsible for fast defense response against various non host

239   fungal pathogens.

240   The primary objective of our study was to evaluate the role of *PEN* genes of Arabidopsis in

241   providing resistance against *R. solani*. Till now, no non-host resistance (NHR) gene has been

242   reported that provides disease resistance against the necrotrophic fungus- *Rhizoctonia solani*

243   with broad range host. We screened the Arabidopsis *PEN* genes, including *PEN1, PEN2* and

244   *PEN3,* among which it was found that *PEN2-3* provides disease resistance against rice sheath

245   blight pathogen *R. solani* for the first time. As per the previous report by Lazniewska et al.

246   (2012), we observed the formation of similar infection cushions in *pen2-3* mutants upon

247   infection with *R. solani.* Unlike *pen2-3*, *pen1* and *pen3-1* mutants did not allow the formation of

248   infection cushion at 30hpi (Fig. 1). In *pen3-1,* the runner hyphae gave rise to swollen hyphal tips

249   instead of forming cushion like structures. This experiment made it quite apparent that there is a

250   clear distinction between hyphal branching formed by wild type and infection cushion formed by

251   *pen2-3*.

252   To dissect the role of *PEN* genes of Arabidopsis in conferring non-host resistance against *R.*

253   *solani* further*,* we performed DAB and Aniline blue staining. The observation from DAB

254   staining clearly indicated the production of H_2O_2 in *pen2-3* at many places where there is site of

255   interaction from cushions of the mycelia and Arabidopsis epidermal cells as compared with

256   *pen1*, *pen3-1* and wild type Col-0. As it is already reported that the production of H_2O_2 not only

257   provide direct defense response but also follows signal transduction pathway which lead to

258   hypersensitive response (HR) (Waetzig et al. 1999). The production of H_2O_2 was not only

259   observed at the site of infection in *pen2-3* but also in the neighboring cells of the inoculated

260   leaves which signifies that neighboring cells defense system has already activated and shows up

261   HR response by following signaling. Contrastingly, *pen1* showed reduced peroxidase activity at

262   the early hour of infection. *pen3-1* showed little higher accumulation of H_2O_2 than *pen1* but less

263   than *pen2-3* (Fig. 2). Thus, higher production of H_2O_2 in *pen2-3* at an early stage may indicate

264   the pathway activating the early signaling event are intact in the mutant.

265   Production of callose; composed of β-(1,3)-glucan polymer, serves as a biomarker against

266   intrusion by pathogens at the site of infection in host (Luna et al. 2011). Callose deposition

267   varied in all the three *pen* mutants. *pen1* failed to produce enhanced callose in response to *R.*

268   *solani* infection. In contrast to *pen2*-3 which showed thick callose deposition at the site of

269   infection, *pen1* showed patch like pattern (Fig. 3). The notable increase in the deposition of

270   callose in *pen2-3* mutant may explain the number and extent of attempted sites of fungal

271   penetration. Previously it is reported that papillae composed of callose are deposited at the sites

272   of penetration into the cell wall by the fungal pathogen, which results in the cell wall thickening

273   (Ellinger et al. 2013).

274   Macroscopic infection symptom results clearly showed the elevation in lesion formation. This

275   results correlates with the increased formation of infection cushions in *pen2-3* (Fig. 4). Further,

276   chlorosis, declination in chlorophyll level, higher necrotic lesions and increased leaf senescence

277   in *pen2-3* mutant leaves suggested that *pen2-3* was most susceptible among the other two *pen*

278   mutants. Confirmation from the infection of complement line of *pen2-3* showed similar behavior

279   as that of Col-0 (Fig. 5).

280   In summary, our study has uncovered the involvement of *PEN2* gene from Arabidopsis at pre-

281   penetration stage which is an early infection process in providing disease resistance against

282   broad host range pathogen *R. solani* which has not been reported previously. Further,

283   characterization of the genes in multiple plant hormone pathway mutants might show clear idea

284   on the involvement of the genes in NHR disease resistance against rice sheath blight.

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Supplementary Figure Legends Information

374 **S1**. **PCR based confirmation of homozygous *PENETRATION* mutants used in the study.** (a)

375 the T-DNA insertion mutant of *pen1* (SALK_004484C) was confirmed using a T-DNA left

376 border primer indicating the presence of insert in the mutant. (b) *pen2-3* was confirmed using a

377 CAPS marker indicating the presence of an additional restriction site for *Bsm*A*I* restriction

378 enzyme. (c) *pen3-1* was also confirmed using a CAPS marker showing deletion of *HphI*

379 restriction site in the mutant.

380 **S2. Infection phenotypes of Arabidopsis wild type and *pen* mutants upon infection with *R.**

381 *solani*.** Leaves of Col-0 and *pen* mutants were stained with trypan blue after 6, 12, 24, 30, 36 and

382 48hpi to observe the extent of disease progression. Stained leaves were observed under bright

383 field microscope. Leaves inoculated with water were used as control. Scale bar = 100μm

384 **S3. Total chlorophyll estimation upon infection with *R. solani* on Arabidopsis wild type**

385 **(Col-0) and *pen* mutants.** Total chlorophyll content measured in leaves of wild type and *pen*

386 mutants of *A. thaliana* after 30 and 48hpi with *R. solani* sclerotia. Leaves treated with water

387 served as control. Data represent the mean ± SEM of three biological replicates ($n = 3$). The

388 experiment was carried out three times with similar results.

389 **S4. SEM micrographs of Arabidopsis *pen* mutants showing post infection hyphal**

390 **colonization by *R. solani* at 30hpi and 48hpi.** Leaves of Arabidopsis wild type (Col-0) and *pen*

391 mutants inoculated with water as control. Col-0 and *pen* mutants inoculated with *R. solani*

392 sclerotia showed disease progression with profuse hyphal branching after 30hpi. *pen2-3* showed

393   sporadic onset of infection cushion formation (ioc; dashed arrow) after 30hpi. Col-0 and *pen*

394   mutants inoculated with *R. solani* sclerotia observed after 48hpi. Dense infection cushions (ic;

395   solid arrows) at 48hpi in *pen2-3*. Swollen hyphal tip formed lobate apperesoria (la) at 48hpi in

396   *pen3-1*. Scale bar ~ 50µm

397   **S5. Macroscopic quantification of disease severity of *R. solani* infected Arabidopsis *pen***

398   **mutants.** Macroscopic necrotic lesions were quantified in terms of area using ImageJ software

399   after 1, 2 and 3dpi. Data represent percentage mean area ± SEM (n=5).

400   **Supplementary Table 1: List of primers used in the study.**

401   Figure Legends

402   Fig.1. **Formation of infection cushion upon infection with *Rhizoctonia solani* on Arabidopsis**

403   **wild type (Col-0) and *pen* mutants.** Leaves of wild type and pen mutants of Arabidopsis were

404   stained with trypan blue after 30hpi and 48hpi. Maximum cushions formed at 48hpi in *pen2-3*.

405   Infection cushions are indicated by arrows. The experiment was carried out three times, and each

406   contained three biological replicates. Scale bar ~ 50µm.

407   Fig.2. **DAB staining.** Leaves of wild type and pen mutants of Arabidopsis were stained with

408   DAB after 30hpi and 48hpi. Production of H_2O_2 was observed at the site of infection as

409   yellowish-brown precipitate. Each experiment was carried out with three biological replicates.

410   Scale bar-50µm.

411   Fig.3. **Accumulation of callose at sites of infection in Arabidopsis leaves.** Leaves of wild type

412   and *pen* mutants of Arabidopsis were stained with aniline blue after 30hpi and 48hpi. Callose

413   deposition was observed UV excitation of a fluorescence microscope. Scale bar = 50µm.

414   Fig.4. **Macroscopic quantification of disease progression in Arabidopsis wild type and *pen***

415   **mutants.** Four week old plants were infected with *R. solani* sclerotia and photographed at 0dpi,

416   1dpi, 2dpi and 3dpi. Leaves inoculated with water were used as control. The coverage of necrotic

417   lesions increased with time in each accession with *pen2-3* being the most affected. The

418   experiment was carried out three times, and each contained three biological replicates.

419   Fig.5. **Comparison of infection structure of *PEN2-GFP* and *pen2-3* as compared to wild**

420   **type Col-0.** Leaves of wild type Col-0, PEN2-GFP and *pen2-3* mutants of Arabidopsis were

421   stained with trypan blue after 30hpi and 48hpi. Maximum cushions formed at 48hpi in *pen2-3*.

422   Infection cushions are indicated by arrows. Scale bar ~ 50μm.

423

Figure 1

Formation of infection cushion upon infection with *Rhizoctonia solani* on Arabidopsis wild type (Col-0) and *pen* mutants
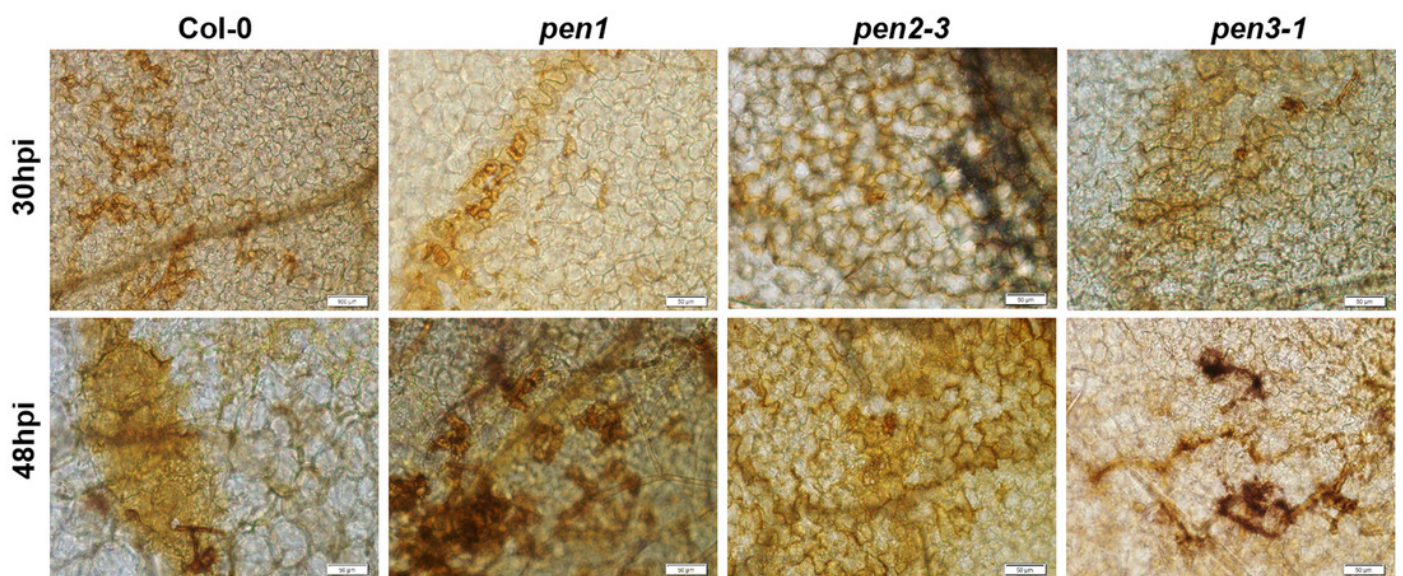
Leaves of wild type and pen mutants of Arabidopsis were stained with trypan blue after 30hpi and 48hpi. Maximum cushions formed at 48hpi in *pen2-3.* Infection cushions are indicated by arrows. The experiment was carried out three times, and each contained three biological replicates. Scale bar ~ 50µm.
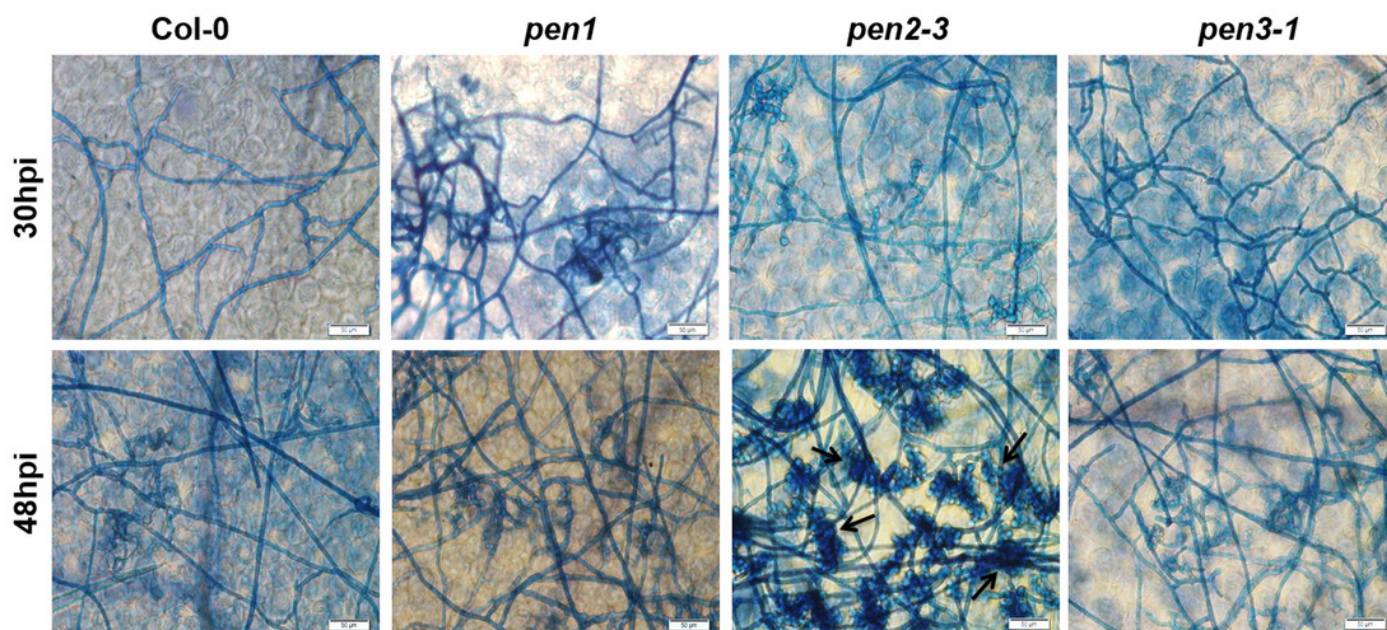
Figure 2

DAB staining

Leaves of wild type and pen mutants of Arabidopsis were stained with DAB after 30hpi and 48hpi. Production of H_2O_2 was observed at the site of infection as yellowish-brown precipitate. Each experiment was carried out with three biological replicates. Scale bar-50µm.
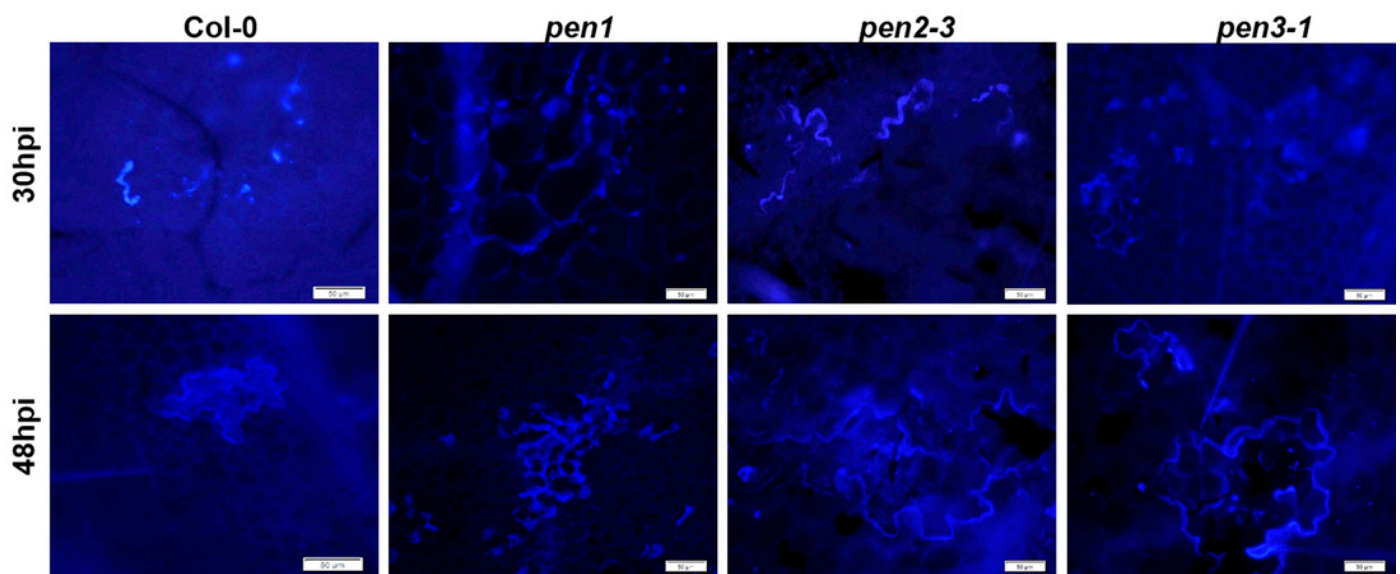
Figure 3

Accumulation of callose at sites of infection in Arabidopsis leaves

Leaves of wild type and *pen* mutants of Arabidopsis were stained with aniline blue after 30hpi and 48hpi. Callose deposition was observed UV excitation of a fluorescence microscope. Scale bar = 50μm.
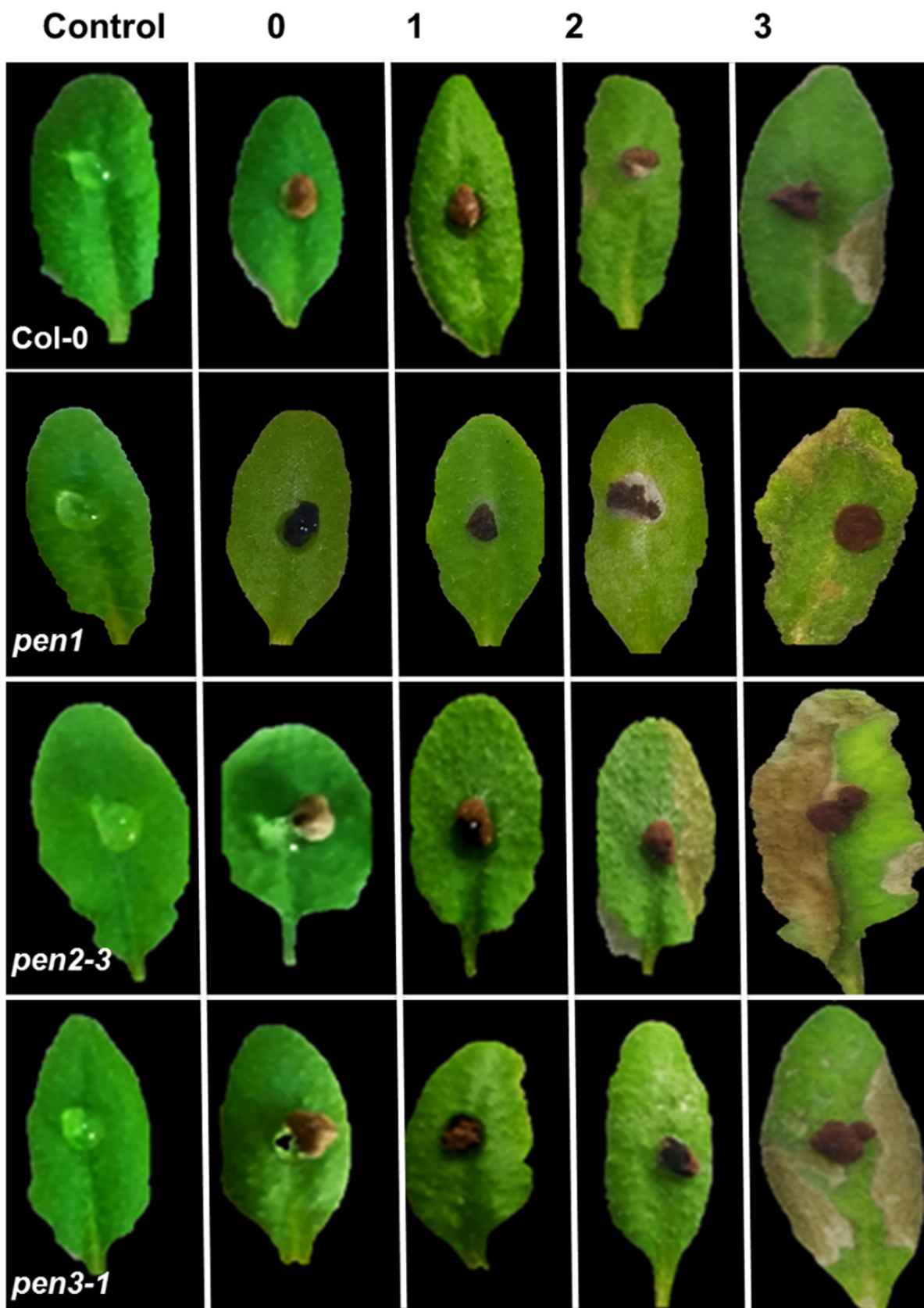
Figure 4

Macroscopic quantification of disease progression in Arabidopsis wild type and *pen* mutants

Four week old plants were infected with *R. solani* sclerotia and photographed at 0dpi, 1dpi, 2dpi and 3dpi. Leaves inoculated with water were used as control. The coverage of necrotic lesions increased with time in each accession with *pen2-3* being the most affected. The experiment was carried out three times, and each contained three biological replicates.

Days of post inoculation (dpi)

Figure 5

Comparison of infection structure of *PEN2-GFP* and *pen2-3* as compared to wild type Col-0

Leaves of wild type Col-0, PEN2-GFP and *pen2-3* mutants of Arabidopsis were stained with trypan blue after 30hpi and 48hpi. Maximum cushions formed at 48hpi in *pen2-3.* Infection cushions are indicated by arrows. Scale bar ~ 50μm.