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

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*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*. 
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Abstract

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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,
maize and sorghum; aerial blight and stem rot in legumes like mung bean and soybean; sheath rot in sugarcane, damping off of cotton, black scurf and sprout canker in potato, heart rot in cabbage, and foliar blights in other fruits and plantation crops (Ajayi-Oyetunde & Bradley 2018; Nagaraj et al. 2017). Rice sheath blight is the most devastating disease challenging global food security amongst other diseases caused by \textit{R. solani} and can potentially cause around 50% reduction in rice yield worldwide (Zheng et al. 2013). \textit{R. solani} is both soilborne as well as waterborne pathogen. This non-sporulating fungus survives in the form of sclerotia during the inactive phases of its infection cycle (KUMAR¹ et al. 2009). Under favorable conditions, these sclerotias germinate into mycelia which further form atypical hyphal aggregates called infection cushions, required for host penetration (Kumar et al. 2011; Łażniewska et al. 2012; Singh et al. 2012; Taheri & Tarighi 2011). \textit{R. solani} induces programmed cell death with loss of photosynthetic activity and development of necrotic lesions in the host tissues (Taheri & Tarighi 2011) (Mondal et al. 2012). It is also reported that the hyphal growth and penetration of \textit{R. solani} is influenced by protrusions or openings on the leaves of host surface, such as trichomes, stomata or papillae (Basu et al. 2016).

Most of the plant diseases are caused by fungal pathogens (Łażniewska et al. 2012). To fight against various pathogenic attacks, plant possess immune system with multilayered continuum of both pre-formed and acquired barriers. The disease resistance is mediated by sequential basal and resistance (R)-gene mediated hypersensitive response that does not always involve recognition of pathogenic cues (Gill et al. 2015). Non-host resistance demarcates the host range of phytopathogenic microorganisms, representing the hallmarks of basic compatibility. Thus, adapted pathogens always try to suppress or evade the plant's basal defense mechanism by secreting a repertoire of effector molecules (Speth et al. 2007) against robust and durable nature
of NHR as a part of innate immunity (Nuernberger & Lipka 2005). Durability of NHR has increased attention to revamp resistance in crop. Elaborated suite of plant defense system induces downstream cell-autonomous responses of PAMP-triggered immunity (PTI) including production of reactive oxygen species (ROS), MAP-kinase signaling, transcriptional induction of pathogenesis-related (PR) genes, and callose deposition (Bittel & Robatzek 2007).

In Arabidopsis, cell wall based defenses are mediated by three *PENETRATION* genes - *PEN1*, *PEN2* and *PEN3* and allow limited entry of non-adapted powdery mildews. *PEN1* encodes a syntaxin (SYP121/PEN1) which belongs to the SNARE superfamily proteins. *PEN1* plays a role in the papilla formation (Collins et al. 2003). *PEN2* encodes myrosinase, associated with peroxisomes, which implicates production of glucosinolate derivatives as an antifungal defense compounds (Bednarek et al. 2009; Lipka et al. 2005). The toxic by-products of *PEN2* are transported to the sites of pathogen entry by ABC transporter proteins which is encoded by *PEN3* gene. *PEN2* and *PEN3* have been documented to confer disease resistance against biotroph *viz.* *Erysiphe pisi*, hemibiotrophic oomycete *Phytophthora infestans* as well as necrotroph *P. cucumerina* (Stein et al. 2006).

The molecular mechanism of disease resistance to *R. solani* and its mode of infection in hosts are not clear. Therefore, the objective of this study was to unravel the defense strategies against *R. solani* in non-host wild type Arabidopsis Col-0 and *pen1, pen2-3* and *pen3-1* mutants.
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
14 hour photoperiod with ~100 µE/m²/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, H₂O₂ 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
acid: water – 1:1:1:1, v/v). It was further destained with lactophenol, mounted onto glass slide with 50% glycerol and examined under bright field microscope. For DAB staining, infected leaves were incubated in 1mg/mL aqueous DAB solution for 8h in dark following which stain was replaced with water and incubated in similar conditions. Destaining was performed with acetic acid: ethanol (96:4 v/v) and mounted with 50% glycerol and visualized under brightfield microscope. For aniline blue staining, leaves were cleared in alcoholic lactophenol and stained overnight with 0.01% aniline blue in 150mM K$_2$HPO$_4$, pH 9.5. Stained leaves were equilibrated with 50% glycerol and observed under UV excitation.

**Extraction and Estimation of Chlorophyll content**

One gram of treated and untreated leaves were taken at 30 and 48hpi and ground with 20 mL of 80% acetone. It was then centrifuged at 5000 rpm for 5 minutes. The supernatant was collected in a tube and the process was repeated until the residue appears colorless. Supernatant was made up to 100 mL with 80% acetone. The absorbance of the solution was recorded at 645 nm and 663 nm against the blank solvent (acetone) (Rajalakshmi & Banu 2013). The concentrations of chlorophyll a, chlorophyll b and total chlorophyll was measured as mg/g of the sample and were calculated as described by Rafii et al. 2015 (Azizi et al. 2015) using the following equation:

$\text{Chlorophyll a (mg/g Fresh leaf)} = 12.7 \times (A_{663}) - 2.69 \left(\frac{A_{645}}{1000}\right) \times \frac{V}{W}$

$\text{Chlorophyll b (mg/g Fresh leaf)} = 22.9 \times (A_{645}) - 4.68 \left(\frac{A_{663}}{1000}\right) \times \frac{V}{W}$

$\text{Total Chlorophyll (mg/g Fresh leaf)} = 20.2 \times (A_{645}) + 8.02 \left(\frac{A_{663}}{1000}\right) \times \frac{V}{W}$
Infected leaves were fixed in 4% paraformaldehyde and subsequently washed with phosphate buffer. Leaves were blotted dry with kimwipe and mounted on metal stubs for visualization under environmental SEM. Each plant had a number of three detached leaves and data from three biological replicates.

**Pathogenicity Assays and calculation of disease level using detached leaves**

The infected rosette leaves from the wild type and *pen* mutants, harvested at 1dpi, 2dpi and 3dpi were photographed. The percentage of disease level was calculated based on the area of necrotic lesions formed at different time point in all accessions of Arabidopsis. At 1dpi, 2dpi and 3dpi, disease level was calculated in whole plant as follows:

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

Each plant had a number of three detached leaves and data from three biological replicates were taken for calculation. The data were expressed as means ± standard error of the mean (SEM).

**Results**

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

In order to assess the resistance phenotypes in Arabidopsis wild type Col-0 and penetration deficient mutants, a T-DNA insertion mutant *pen1* and EMS generated *pen2-3* and *pen3-1* were used. The *pen* mutants obtained from NASC were confirmed for their homozygosity by using PCR based analysis (Fig. S1, Supplementary Table 1). Leaves of four weeks old plants were challenged with *R. solani* sclerotia for 6, 12, 24, 30, 36 and 48 hours post inoculation (hpi). We
observed no mycelial growth after 6 and 12hpi in either Col-0 or the *pen* mutants. However, at 24hpi and 30hpi, onset of branching of secondary hyphae were observed in Col-0 but no such extensive mycelial colonization was observed (Fig. S2). On the other hand, *pen* mutants showed profuse branching of the fungal hyphae. Among the *pen* mutants, *pen2-3* showed maximum fungal colonization and initiation of typical infection cushions was observed at 30hpi, which lacked in wild type as well as *pen1* and *pen3-1*. Prominent infection cushions were clearly visible at 36hpi and 48hpi in *pen2-3*, which was initiated in *pen3-1* at 48hpi and totally absent in Col-0 and *pen1* (Fig. S2). To further examine the results obtained in previous time course experiment, we focused our observations on 30hpi where initiation of typical infection cushion was seen in *pen2-3* and 48hpi where well-formed infection cushions were observed (Fig. 1). Leaves of *pen2-3* showed several runner hyphae entangled together to form lobate appressoria and initiation of infection cushion at 30hpi. At 48hpi, there was more hyphal proliferation in *pen2-3* which resulted in formation of compact bundles of fungal hyphae entrenched onto the leaves surface and the formation of infection cushions (Fig. 1).

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

Pathogen virulence leads to the inhibition of photosynthesis (Okorski et al. 2008). Thus, the disappearance of chlorophyll was analyzed at 30 and 48hpi. Compared to the untreated leaves of Arabidopsis, treated leaves showed reduced chlorophyll content. Higher amount of chlorophyll content was recorded in treated leaves of Col-0 with that of *pen* mutants. *pen2-3* showed maximum degradation in cellular chlorophyll content as compared to wild type, *pen1* and *pen3-1* 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$_2$O$_2$ 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$_2$O$_2$ was detected as dark yellowish-brown precipitate (Fig. 2). As expected, *pen2-3* exhibited relatively higher level of accumulation of H$_2$O$_2$ 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.
Macroscopic lesion phenotypes induced by *R. solani* vary between wild type and *pen* mutants of Arabidopsis

Wild type Col-0 and the *pen* mutants were almost indistinguishable up to 1dpi (days of post inoculation) with sclerotia of *R. solani*. The wild type became chlorotic upon increasing the inoculation time up to 3dpi and did not develop much lesions spontaneously. In contrast to that *pen1*, *pen2-3* and *pen3-1* started showing necrotic lesions along with chlorosis at 2dpi. *pen2-3* showed maximum necrotic lesion and chlorosis at 2dpi and 3dpi. Macroscopic lesions were almost identical up to 1dpi. By contrast, when the infection period was increased up to 3dpi, mutants were clearly distinguished from the wild type, especially as seen in leaves of *pen2-3* mutant (Fig. 4). Based on this experiment, percentage of disease severity was calculated (Fig. S5). Wild type Col-0 had an average of 0.09%, 0.55%, 22.08% necrotic tissues at 1dpi, 2dpi and 3dpi respectively (average ± standard deviation, n= 5). *pen1* showed an average necrotic lesion of about 0.102%, 0.064%, 27.06% at 1dpi, 2dpi and 3dpi respectively. On the other hand, *pen2-3* mutant leaves showed maximum necrotic lesion with an average of 0.139%, 36.38% and 67.54% at 1dpi, 2dpi and 3dpi respectively. Leaves of *pen3-1* showed relatively lesser necrotic lesions than *pen2-3* but more than *pen1* and Col-0 with an average of 0.108%, 2.71% and 43.54% at 1dpi, 2dpi and 3dpi respectively.

*PEN2* provides penetration resistance against *R. solani*

To authenticate the result that *pen2-3* mutant triggers cushion formation, we targeted complement line expressing green fluorescent protein (GFP)-*PEN2*, driven by native 5' regulatory region in *PEN2-1* background. Due to the knockdown of *PEN2*, *pen2-3* allowed the formation of infection cushions on the leaves surface. Furthermore, the complemented line (PEN2-GFP) for the *pen2-3* mutant, behaved similar to wild type Col-0 with no cushion
formation at 30hpi and 40hpi in response to *R. solani* (Fig. 5). This experiment corroborated our hypothesis that *PEN2* enhance penetration resistance against *R. solani*.

**Discussion**

For a successful plant-pathogen interaction, the pathogen recognizes a surface for the initiation of its growth and further penetrates into the host tissue to cause infection. Non-host resistance provides durable protection against invading pathogens by using various defense strategies (Thordal-Christensen 2003). The coherent investigation of our present study revealed that *pen2-3* contributes to non-host resistance in *Arabidopsis thaliana* to *Rhizoctonia solani*, a necrotrophic rice pathogen. Among the *pen* mutants, including *pen1, pen2-3 and pen3-1, pen2-3* was observed with compromised penetration resistance to *R. solani*. *Pen2-3* showed maximum number of atypical infection cushions which clearly depicts the important role playing in non-host resistance against sheath blight in rice.

Flentje (1963) reported that the infection cushions develops only in the susceptible host which is suppressed in the resistant host (Flentje et al. 1963). Despite having very less in-depth studies about the mechanism of infection process of *R. solani* in various host, it is reported that *R. solani* produces typical infection structure by forming cluster of hyphae with bulbous end during its pre-penetration stage (Łaźniewska et al. 2012). This clustering T-shaped branched hyphae and formation of infection cushions further leads to penetration by defeating the barriers present in the host tissue, enters via penetration peg and promotes colonization (Pannecoucque & Höfte 2009). To fight against the invasion of pathogens, *Arabidopsis* have penetration genes (*PEN*) which avoid penetration and is responsible for fast defense response against various non host fungal pathogens.
The primary objective of our study was to evaluate the role of PEN genes of Arabidopsis in providing resistance against *R. solani*. Till now, no non-host resistance (NHR) gene has been reported that provides disease resistance against the necrotrophic fungus *Rhizoctonia solani* with broad range host. We screened the Arabidopsis PEN genes, including PEN1, PEN2 and PEN3, among which it was found that PEN2-3 provides disease resistance against rice sheath blight pathogen *R. solani* for the first time. As per the previous report by Lazniewska et al. (2012), we observed the formation of similar infection cushions in pen2-3 mutants upon infection with *R. solani*. Unlike pen2-3, pen1 and pen3-1 mutants did not allow the formation of infection cushion at 30hpi (Fig. 1). In pen3-1, the runner hyphae gave rise to swollen hyphal tips instead of forming cushion like structures. This experiment made it quite apparent that there is a clear distinction between hyphal branching formed by wild type and infection cushion formed by pen2-3.

To dissect the role of PEN genes of Arabidopsis in conferring non-host resistance against *R. solani* further, we performed DAB and Aniline blue staining. The observation from DAB staining clearly indicated the production of H$_2$O$_2$ in pen2-3 at many places where there is site of interaction from cushions of the mycelia and Arabidopsis epidermal cells as compared with pen1, pen3-1 and wild type Col-0. As it is already reported that the production of H$_2$O$_2$ not only provide direct defense response but also follows signal transduction pathway which lead to hypersensitive response (HR) (Waetzig et al. 1999). The production of H$_2$O$_2$ was not only observed at the site of infection in pen2-3 but also in the neighboring cells of the inoculated leaves which signifies that neighboring cells defense system has already activated and shows up HR response by following signaling. Contrastingly, pen1 showed reduced peroxidase activity at the early hour of infection. pen3-1 showed little higher accumulation of H$_2$O$_2$ than pen1 but less
than pen2-3 (Fig. 2). Thus, higher production of H$_2$O$_2$ in pen2-3 at an early stage may indicate the pathway activating the early signaling event are intact in the mutant.

Production of callose; composed of β-(1,3)-glucan polymer, serves as a biomarker against intrusion by pathogens at the site of infection in host (Luna et al. 2011). Callose deposition varied in all the three pen mutants. pen1 failed to produce enhanced callose in response to *R. solani* infection. In contrast to pen2-3 which showed thick callose deposition at the site of infection, pen1 showed patch like pattern (Fig. 3). The notable increase in the deposition of callose in pen2-3 mutant may explain the number and extent of attempted sites of fungal penetration. Previously it is reported that papillae composed of callose are deposited at the sites of penetration into the cell wall by the fungal pathogen, which results in the cell wall thickening (Ellinger et al. 2013).

Macroscopic infection symptom results clearly showed the elevation in lesion formation. This results correlates with the increased formation of infection cushions in pen2-3 (Fig. 4). Further, chlorosis, declination in chlorophyll level, higher necrotic lesions and increased leaf senescence in pen2-3 mutant leaves suggested that pen2-3 was most susceptible among the other two pen mutants. Confirmation from the infection of complement line of pen2-3 showed similar behavior as that of Col-0 (Fig. 5).

In summary, our study has uncovered the involvement of *PEN2* gene from Arabidopsis at pre-penetration stage which is an early infection process in providing disease resistance against broad host range pathogen *R. solani* which has not been reported previously. Further, characterization of the genes in multiple plant hormone pathway mutants might show clear idea on the involvement of the genes in NHR disease resistance against rice sheath blight.
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**Supplementary Figure Legends Information**

**S1. PCR based confirmation of homozygous PENETRATION mutants used in the study.** (a) the T-DNA insertion mutant of *pen1* (SALK_004484C) was confirmed using a T-DNA left border primer indicating the presence of insert in the mutant. (b) *pen2-3* was confirmed using a CAPS marker indicating the presence of an additional restriction site for BsmAI restriction enzyme. (c) *pen3-1* was also confirmed using a CAPS marker showing deletion of *HphI* restriction site in the mutant.

**S2. Infection phenotypes of Arabidopsis wild type and pen mutants upon infection with R. solani.** Leaves of Col-0 and *pen* mutants were stained with trypan blue after 6, 12, 24, 30, 36 and 48hpi to observe the extent of disease progression. Stained leaves were observed under bright field microscope. Leaves inoculated with water were used as control. Scale bar = 100µm

**S3. Total chlorophyll estimation upon infection with R. solani on Arabidopsis wild type (Col-0) and pen mutants.** Total chlorophyll content measured in leaves of wild type and *pen* mutants of *A. thaliana* after 30 and 48hpi with *R. solani* sclerotia. Leaves treated with water served as control. Data represent the mean ± SEM of three biological replicates (*n* = 3). The experiment was carried out three times with similar results.

**S4. SEM micrographs of Arabidopsis pen mutants showing post infection hyphal colonization by R. solani at 30hpi and 48hpi.** Leaves of Arabidopsis wild type (Col-0) and *pen* mutants inoculated with water as control. Col-0 and *pen* mutants inoculated with *R. solani* sclerotia showed disease progression with profuse hyphal branching after 30hpi. *pen2-3* showed...
sporadic onset of infection cushion formation (ioc; dashed arrow) after 30hpi. Col-0 and pen mutants inoculated with R. solani sclerotia observed after 48hpi. Dense infection cushions (ic; solid arrows) at 48hpi in pen2-3. Swollen hyphal tip formed lobate apperesoria (la) at 48hpi in pen3-1. Scale bar ~ 50µm

S5. Macroscopic quantification of disease severity of R. solani infected Arabidopsis pen mutants. Macroscopic necrotic lesions were quantified in terms of area using ImageJ software after 1, 2 and 3dpi. Data represent percentage mean area ± SEM (n=5).

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

Figure Legends

Fig.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.

Fig.2. DAB staining. Leaves of wild type and pen mutants of Arabidopsis were stained with DAB after 30hpi and 48hpi. Production of H₂O₂ was observed at the site of infection as yellowish-brown precipitate. Each experiment was carried out with three biological replicates. Scale bar-50µm.

Fig.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.
Fig. 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.

Fig. 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.
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 $\text{H}_2\text{O}_2$ 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.
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.