An Arabidopsis nonhost resistance gene, IMPORTIN ALPHA 2 provides immunity against rice sheath blight pathogen, *Rhizoctonia solani*

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### Article Info

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**Abstract**

There is neither resistant rice cultivar nor any control measure against *Rhizoctonia solani* AG-1 IA (RS), causal of sheath blight and a major threat to global rice production. Rice is a host and Arabidopsis is a nonhost with underlying nonhost resistance (NHR) gene which is largely untested. Using approaches of forward genetics and tools, cytology, and molecular biology, we identified homozygous mutants in Arabidopsis, mapped the NHR gene, and functionally characterized it in response to RS. *Rss1* was mapped on Ch 4 between JAERI18 and Ch4:9.18 (844.6 Kb) and identified IMPORTIN ALPHA 2 as the candidate *Rss1* gene. We found that breach of immunity in *rss1* by RS activates defense responses whereas photosynthetic pigment biosynthesis and developmental processes are negatively regulated. In addition, a gradual decrease in *Pr1* by 3 dpi revealed that *Rss1* positively regulated early SA-mediated resistance. Whereas increased expression of PDF1.2 by 3 dpi supported switching to necrotrophy, SA-mediated defense in Col-0 leading to immune response. Enhanced expression of AGT8a in *rss1* supported autophagic cell death. IMPA2, IMPA1, and RAN1 function together to provide NHR against RS. These findings demonstrate that IMPA2 provides NHR against RS in Col-0 that evoke SA-mediated early immunity with a potential biotechnological application.

### 1. Introduction

A constant increase in the world’s population to 9.15 billion by 2050 put immense pressure to increase the agricultural food security demand by 60–100% globally (Alexandratos and Bruinsma, 2012). It is a major juggernaut to achieve and fulfill the global food sovereignty with demand wherein rice is the common and most important staple food worldwide. Sheath blight in rice is the second most devastating necrotrophic fungal disease by the causative agent, *Rhizoctonia solani* Kuhn AG1-IA (RS) with varied host range, a severe necrotrophic basidiomycete fungus contributing to a yield loss of around 50% worldwide (Mishra et al., 2020; Molla et al., 2020). The early infection process of RS includes hyphal colonization with atypical “infection cushions” and swollen hyphal tips and necrotic lesion formation for which there has neither been a tolerant variety of rice nor any control measure (Basu et al., 2016). Host-specific toxins effector molecules, AGL1P1, RsIA_NP8, glycosyltransferase, cytochrome C oxidase, and peptidase inhibitor 19 domain produced by RS target the defense signaling pathways leading to successful colonization and enhancing rice sheath blight (Li et al., 2021).

To protect against pathogenic invaders, plants perceive the danger signal such as Pathogen/Danger-associated molecular patterns by using the cell surface pattern recognition receptors leading to activation of pattern-triggered immunity. However, pathogens secrete an arsenal of effectors to subdue the potential immunity generated against them which further leads to activation of cognate R-genes in plants via NLR-mediated immunity which is termed effector-triggered immunity (Ngou et al., 2021). Although the gene-for-gene model with specific R-gene mediated immunity in plants persists, nonhost resistance (NHR) prevents successful colonization against certain pathogens. NHR are multi-gene trait exhibiting defense in all plant species against all races of pathogens and are broad-spectrum and more durable which can be a long-hoped goal of plant pathologists for successfully transferring the NHR gene(s) to the susceptible host (Gill et al., 2015; Mysore and Ryu, 2004; Stein et al., 2006). NHR comprises a two-layered defense mechanism i.e., pre-and post-invasive resistance (Lipka et al., 2005). *NON-HOST 1 (NHO1)* is the first identified NHR gene reported to provide immunity against species of bacteria, *Pseudomonas fluorescens*, *P. syringae pv. tabaci* and *P. syringae pv. phaseolicola*, as well as necrotrophic...
fungus Botrytis cinerea (Kang et al., 2003; Lu et al., 2001). Forward genetics screening revealed the well-known PENETRATION genes of Arabidopsis i.e., PENETRATION1 (PEN1), PEN2, PEN3, and PEN4, involved during pre-invasive resistance, essential in providing NHR against barley powdery mildew pathogens Blumeria graminis and Erysiphe pisi (Micali et al., 2008).

Notably, several genes have shown NHR to different necrotrophic fungi. PEN2 conferred penetration resistance against hemibiotrophic fungus Magnaporthe oryzae (Maeda et al., 2009) and necrotrophic fungus RS (Parween et al., 2021), the causal agents of rice blast and rice sheath blight respectively. The PEN4 (phytochelatin synthase (PCS1), plays its role in heavy metal stress tolerance, also provide pre-invasive NHR against B. graminis and M. oryzae (Hematy et al., 2020; Yamaura et al., 2020). PAD4 and SAG101 have been reported to provide broad-spectrum NHR in Arabidopsis against B. graminis stimulating SA production and antimicrobial molecules (Dongus et al., 2020; Lapi et al., 2020; Lipka et al., 2005). Arabidopsis PSS1 (PHYTOPHTHORA SOJAE SUSCEPTIBLE 1) and PSS30 encoding a glycine-rich protein (GRP) and FOLATE TRANSPORTER1 respectively are required for pre-and post-penetration resistance against Phytophthora sojae and Fusarium virguliforme in field conditions (Kambakam et al., 2021; Sumit et al., 2012; Wang et al., 2018). Mutation in barley ml5 (MILDEW RESISTANCE LOCUS O), Arabidopsis ABCG34I (ABC transporter), different signaling pathways like ein2 and ein3 (ET), pad2, pad3, eds4, eds8 (SA), iop1, coi1 and jai1 (JA) lead to a breach of immunity against the necrotrophic fungus species (Alonso et al., 2003; Denby et al., 2004; Ferrari et al., 2003; Khare et al., 2017; Kiraly, 2002; Solano, 2002; Thomma et al., 1998). Although the molecular mechanism of NHR is not very well understood, the practical application of NHR using transgenic approaches has been tested in field conditions producing disease-resistant crops (Fonseca and Mysore, 2019). QTLS reported against RS may be introgressed to generate resistant rice germplasm which however does not work in field conditions as fungal pathogens develop resistance in due course of time (Yellareddygari et al., 2014).

Since rice is susceptible to RS and Arabidopsis is a nonhost with the active defense during early infection, it is possible to identify the underlying NHR gene. To address this, we generated rss1 in Col-0 background with compromise in early immunity against RS. Here, we mapped RSS1 on the southern arm of Chromosome 4 between JAERI18 and CH4.9.16 and identified it which encodes an IMPORTIN ALPHA 2 (IMP2A). We observed that rss1 showed severe HR and cell death upon RS infection which affected in negative biogenesis of the photosynthetic pigments. Furthermore, IMP2A governs SA-mediated early immunity, and rss1 leads to RS-induced autophagic cell death. The result suggests that IMP2A activates the HR and does not allow the establishment of infection structure in Arabidopsis thereby confers early immunity to RS.

2. Materials and methods

2.1. Growing conditions and accessions

Arabidopsis seeds of wild type, Col-0 (N1093) and T-DNA insertion lines impa-2 (SALK_017914 and SALK_099707), impa-1 (SALK_082616), ran1-1 (SALK_138,680), and ABC1 (SALK_045739) were obtained from NASC, Europe. The seeds were sown on flats containing agro peat and vermiculite (3:1), covered with plastic wrap, and stratified for 1 day at 4 °C before transferring to plant growth chamber. Seedlings of rice and Arabidopsis accessions were grown as described previously (Parween et al., 2021).

2.2. RS culture condition and inoculation assay

RS AG-1 1A isolate was cultured regularly on freshly prepared potato dextrose agar (PDA) medium and kept at 28 °C for ~14 days in dark conditions. The sclerotia development was visible at 7-day old culture on Petri dishes. Spectinomycin (100 µg/ml) was used to avoid any bacterial contaminations.

To study the infection pattern and development of RS mycelia in rice and Arabidopsis, detached leaf and in planta infection assay was performed as mentioned earlier (Parween et al., 2021). Briefly, the detached leaves of the 4th leaf stage of rice and 24– old Arabidopsis seedlings were infected with equi-sized sclerotia (2–4 mm in diameter) from the 10-day old RS grown PDA plates. The infected leaves were placed in a Petri plate on sterile water moistened filter paper and covered to maintain high humidity until used for the study.

2.3. Generation of Arabidopsis mutants and mapping population

Around 25,000 Col-0 seeds (M0) were mutagenized by treating with various concentrations of ethyl methane sulphonate (EMS at 0.3%) for 12 h following Qu and Qin (2014). M1 seeds (mutated seeds from above) were sowed onto soil and grown by maintaining the growth conditions as above. The mutated seedlings showed phenotypes like stunted growth and dis-coloration pattern of leaves which might be resulting from a mutation in the gene(s) across the genome. Further, M1 plants were grown to get M2 seeds for the test of homozygosity of susceptible phenotype by infecting the detached leaves with RS that causes rice sheath blight.

To generate an F2 population to map the mutated gene, we chose the resistant wildtype ecotype of Arabidopsis (Ler-0) and crossed it with homozygous susceptible M2 plants. F1 seedlings were confirmed using SSLP markers (NGA106 and LGUSSL671). F2 seeds harvested from true F1 seedlings, and the population thus generated was used for disease scoring and mapping the gene.

2.4. Staining and microscopy analysis

Trypan blue, DAB, and aniline blue staining were performed as described earlier by Park et al. (2009). Stained leaves were observed under brightfield, and fluorescence microscope images were recorded for screening. Determination of H2O2 accumulation (ROS) in Arabidopsis leaves was also determined using 2’, 7’-dichlorodihydrofluorescein diacetate (H2DCFDA) - mediated fluorescence, as previously described by Zeng et al. (2015). Determination of superoxide accumulation was done using nitroblue tetrazolium (NBT) where formazan (dark blue stain) is formed upon reaction with superoxide (Fryer et al., 2002). Infected leave samples were also stained with Calcofluor White stain (18,909 Merck), which binds to fungal chitin and check the progression of RS hyphae. Infected leave samples were placed on a clean glass slide and a drop of Calcofluor White stain followed by a drop of 10% KOH solution was added. It was allowed to incubate for 1–2 min and was examined under a confocal microscope (Leica TCS SP8).

2.5. Live cell photosynthetic pigments imaging and chlorophyll estimation

Live cell imaging of the chloroplast autofluorescence was measured under a Laser scanning live-cell imaging microscope (Leica, STELLARIS 5) with an excitation of 633 nm and emission of 647–721 nm (Irieda and Takano, 2021). Chlorophyll (chl) estimation in the infected leaves was performed as elaborated in Parween et al. (2021).

2.6. Electrolyte leakage assay

Electrolyte leakage assay was performed as previously described by Jamra et al. (2021). Briefly, fifteen leaves from 24th-day-old seedlings were challenged with RS. Freshly harvested infected detached leaves discs (15 numbers) that were submerged in 10 mL of sterile water and shaken gently and the membrane leakage (electrolytes (ions) (µΩ−1) leaked (IL initial) was measured by a conductivity meter from the. The samples were then subjected to boiling in a water bath for 15 min and final conductance was measured (IL Final). Percentage (%) of ion leakage was calculated as...
2.7. Mapping of NHR gene

Genomic DNA was isolated from the screened individual seedlings of the F₂ population using the CTAB method (Lukowitz et al., 2000). An equal amount of genomic DNA was pooled from F₂ lines to make separate bulks of S₁ (moderately susceptible), S₂ (highly susceptible), and R (resistant) for bulk segregation analysis (BSA). PCR was done using individual bulked lines to map the possible region of the candidate gene responsible for disease phenotype. We used simple sequence length polymorphism (SSLP), Cleaved amplified polymorphic sequences (CAPS), and sequence-based polymorphic (SBP) markers that are evenly distributed throughout the genome of Arabidopsis. Additional SBP markers were designed as described earlier (Sahu et al., 2012). Markers used for PCR analysis are mentioned in Table S5. Bulked S₂ DNA (100 ng) was then sent for Illumina sequencing (3i Molecular solutions, Bangalore, India). FASTQ data obtained from the next-generation sequencing (NGS) were converted to BCF format (.bcf) using SIMPLE sequencing (NGS) were converted to BCF format (.bcf) using SIMPLE pipeline (http://bar.utoronto.ca/NGM) to visualize, give SNP calls and analyze the data (Austin et al., 2011).

2.8. RNA extraction and RT-qPCR analysis

Total RNA was extracted from the mock and infected leaf samples of rice and Arabidopsis accessions at different inoculation times using the TRIzol® reagent (Invitrogen). Purity and concentration of RNAs were then quantified using Nanodrop (Bio spectrophotometer, Eppendorf) and 1% agarose gel electrophoresis. Further, DNase I (Novagen, Millipore) treatment was performed to get rid of genomic DNA which was confirmed by performing a -RT-qPCR reaction using AtACTIN2 primer pair. RNA samples were then used to synthesize cDNAs at 25 °C for 10 min, 42 °C for 60 min, and followed by 75 °C for 15 min using a Thermo Scientific cDNA synthesis kit. The expression level of SA- (AtPR1, AtICS1), JA (AtPDF1.2a), leaf senescence (AtNHL10), autophagy (AtATG8), and AtMPA2 marker genes were estimated which was normalized to the expression of AtACTIN2 as an internal control. The expression level in rice was analyzed using gene-specific primers of OsPR1, OsPDF1A and normalized with OsActin. Primers used in this study are mentioned in Table S6. qPCR was performed using SYBR Green Master Mix (Bio-Rad) in a Real-Time system (Bio-Rad) and relative expression levels were calculated using the 2⁻ΔΔCt method (Pfaffl, 2001).

2.9. Statistical and in silico analysis

For the detached leaf infection assay, a minimum of three leaves per plant and 3 seedlings of each Arabidopsis accessions and rice (Swarra) were used. Each experiment was conducted with three biological replicates and similar results were obtained in three independent experiments. All the graphs represent mean (± SD) where statistical significance analysis was performed using Student’s t-test for two groups and two-way ANOVA for multiple comparisons using GraphPad Prism 7. Relative intensities of trypan blue, DAB, H₂DCFDA were calculated using GIMP 2.10.10 (Scalschi et al., 2015). ImageJ: IJ 1.46r (Schneider et al., 2012) was used to measure the necrotic lesions for disease severity. Domain prediction using the deduced protein sequence was performed at the SMART online tool (Letunic et al., 2020).

3. Results

3.1. Identification of RS-susceptible Arabidopsis mutant with compromise in immunity

Arabidopsis (Col-0) is a nonhost to RS (Parween et al., 2021). The presence of NHR genes providing immunity against RS in Col-0 is preclusive to the disease occurrence. To find the putative resistance genes, seeds of Col-0 were mutagenized by 0.3% (w/v) EMS. The M₁ progeny clearly showed dis-coloration of leaflets (Fig. S1A) and stunted growth (Fig. S1B). M₁ seeds were harvested individually and stored at 4 °C until further use. We screened the detached leaves from the M₂ seedlings for susceptible responses by infecting them with RS sclerotia. Further, the homozygous mutants were isolated in M₃ generation and named the most susceptible plant as Rhirozonia solani susceptible 1 (rs11). The pathogenicity of RS at 48-h post-inoculation (dpi) was also validated in the rs11 plants through trypan blue (Fig. S1C), DAB (Fig. S1D), and aniline blue (Fig. S1E) staining.

The pathogenicity of RS in rs11 with a susceptible response as early as 30 hpi was depicted by infection cushion was confirmed by trypan blue (Fig. 1A) and accumulation of H₂O₂ as marked by brown precipitate from DAB staining (Fig. 1B). Trypan blue stained rs11 leaves infected by sclerotia of RS showed heavy mycelia with branched atypical infection cushions and maximum colonization as compared to Col-0 which showed perpendicular runner hyphae. DAB staining showed a significantly higher accumulation of H₂O₂ in rs11 at infection sites as compared to Col-0. Graphical representation of trypan blue and DAB intensities showed significantly high stain uptake in rs11 as compared to Col-0 (Fig. 1C).

It was therefore of interest to compute the cell death from detached leaves of Col-0 and rs11 challenged with RS at 30 hpi through ion leakage assay. The RS-treated leaves of rs11 showed enhanced cell death as compared to Col-0 where the ion leakage values (µΩ s) obtained were normalized to the respective water control (Fig. 1D). The severity of RS on rs11 was also validated through in planta infection assay using similar sizes of sclerotia and disease phenotypes were observed at 0, 3, and 6 dpi (Fig. 1E). The rs11 exhibited compromised immunity to the nonhost pathogen, RS, that showed a higher disease lesion area as compared to Col-0 with increasing time points.

3.2. Molecular mapping of RSS1 by bulk segregated analysis and its identification via NGS analysis

To identify the putative mutated gene responsible for RS pathogenicity in rs11, we followed the bulked segregant analysis, and rs11 was crossed with Ler-0 (resistant ecotype of Arabidopsis). The true F₂ seedlings produced from the above outcross were confirmed genotypically by using NGA106 (Fig. S2A) and LUGSSLP671 (Fig. S2B). F₂ population thus generated was screened further with RS, followed by trypan blue staining, and their infection phenotypes were observed under a bright-field microscope. Subsequent bulking of F₂ progeny was done based on the disease phenotype screening against RS. They are denoted as resistant (R), mycelia growing over leaf surface with the initiation of infection cushions (S1) and leaves with infection cushions and profuse mycelial branching (S2), representing low, medium, and highly susceptible phenotype (Fig. S2C). Thirteen out of 54 F₂ seedlings were found to be super-susceptible (S2). Screened infected leaves of the F₂ population showed a 3:1 Mendelian inheritance ratio, indicating a single recessive gene, RSS1 controlling the NHR in Col-0 (Table S1). We pooled the genomic DNA from the thirteen susceptible seedlings and performed the bulked segregant analysis using evenly placed molecular markers (SSLP, CAPS, and SBP) across all the 5 Chromosomes (Figs. S4–S8). It was found that the RSS1 putatively maps to the lower arm of Chromosome 4 flanked by JAEIR18 and TGSSL2P. Fine mapping of the F₂ population confines the map location of RSS1 to 844.6 Kb that is localized between JAEIR18 and CH49_0.18 (Fig. 2A; Table S2).
NGM analysis was based upon discordant chastity (Chs) of the southern arm of Chromosome 4 showed non-recombinant (Fig. 2 B). The abundance over each chromosome, and we found that the EMS mutation mapping). Genome-wide filtered SNPs were plotted based on the reference genome. The pooled genomic DNA was sent for -NGS and data obtained were run via SIMPLE pipeline upon trimming of the adaptors and aligned to TAIR-10 reference genome. The pooled genomic DNA was sent for -NGS and data obtained were run via SIMPLE pipeline upon trimming of the adaptors and aligned to TAIR-10 reference genome. The rsl1.bcf.ngm file thus generated was used to visualize the positional mapping of the candidate gene responsible for RS susceptibility at the online tool called NGM (Next-generation EMS mutation mapping). Genome-wide filtered SNPs were plotted based on their abundance over each chromosome, and we found that the southern arm of Chromosome 4 showed non-recombinant (Fig. 2 B). The NGM analysis was based upon discordant chastity (Chs) value of 1 which comprehends the expected homozygous mutated allele as compared to the reference genome. Also, the chastity threads were generated by applying a higher k-value (k-means clustering, k = 7) which increases the homozygous to heterozygous ratio, and smaller kernel size (kernel density=0.25) to refine and validate the candidate SNPs responsible for the causal phenotype (Fig. 2 B, C). From the NGM mapping tool, we found two SNPs leading to nonsynonymous mutations (Table S3) at the mapped region on Chromosome 4. We screened all the respective T-DNA insertion lines SALK_099707 as rsl1–1 and SALK_017914 as rsl1–2. The molecular mapping, NGM analysis of the NGS data, and early hour infection assays suggest that a non-synonymous mutation of AT4G16143 (IMPA2) in rsl1 compromised penetration resistance in Col-0 against RS. We aligned all 9 paralog protein sequences of Arabidopsis. IMPA2 had 85.63% similarity with IMPA1 (At3g06720) and 6 rice orthologs where IMPA2 has shown 80.50% similarity with importin subunit alpha-1a-like (Os01g14950) and 79.43% with importin subunit alpha-1b (Os05g06350.1) (Table S7). Further, the mutated proline at 65th position in rss1 was found to be conserved in IMPA2 and we speculated the similar functionality of the paralog in providing defense against RS.

3.3. Sheath blight resistance in Arabidopsis is conferred by the IMPA2

We evaluated the disease severity during nonhost-RS (Col-0-RS) interaction based on macroscopic symptoms and lesion sizes as compared to that of host-RS (Rice- RS) (Fig. 3 A–C). Gradual increase in chlorotic and necrotic lesions were observed in RS challenged rice (Swarna) leaves at 1, 2, and 3 dpi (Fig. 3A). Col-0 was resistant with much lesser or no signs of visual necrotic lesion up to 3 dpi at the infection site whereas leaves of rss1, rss1–1, and rss1–2 showed similar necrotic lesions (banded krait) like rice (Fig. 3B). The percentage of
with adenine (A) leading to encoding the zoom-in pictures. Stained leaves were observed under a bright-field microscope. Infection cushions in T-DNA insertion lines to that of trypan blue stained leaves at 2 dpi showed the development of similar atypical comparison to the identified homozygous rss1. Microscopic observations of the putative T-DNA insertion lines (SALK_099707 as homozygous belt to derive the nonsynonymous SNPs (Table S3). (D) Screening of two gosity chastity belts across the Chromosome 4 by keeping the CD value to be 1. Fig. 2. Parween and B.B. Sahu. (B) NGM view of the aligned NGS data which depicts the homozygous rss1 on Chromosome 4 via NGM analysis. (A) Mapping of RSS1 onto Chromosome 4 between JAERI118 and CH14 9.18. (C) The window highlights the region onto the Chromosome 4 of the homozygous belt to derive the nonsynonymous SNPs (Table S3). (D) Screening of two T-DNA insertion lines (SALK_099707 as rss1–1 and SALK_017914 as rss1–2) of the putative RSS1 independently for disease phenotype as above with a comparison to the identified homozygous rss1. Microscopic observations of the trypan blue stained leaves at 2 dpi showed the development of similar atypical infection cushions in T-DNA insertion lines to that of rss1.1. Inset figures depicts the zoom-in pictures. Stained leaves were observed under a bright-field microscope. Bar=100 μm. (E) The schematic representation of RSS1 (At4g16143; encoding IMPORTIN ALPHA 2) identified with a substitution of guanine (G) with adenine (A) leading to 65S. The places of two T-DNA insertion Arabidopsis accessions with different target sites in IMPA2 gene (marked by black shaded triangles) and site of EMS generated point mutation (marked by a black star) in the first exon. (F) Schematic diagram of the domains predicted online. The nonsynonymous mutation in the IBB domain at the 65th position is depicted. The underneath amino acid span length corresponds to specific domains with predicted E-value are tabulated.

Disease severity was calculated based on the disease spread from the observed visible browning lesions on leaves with a necrotic lesion at 3 dpi in rss1 was highest (Fig. 3C). To assess RS-induced macroscopic lesion resulting from the HR process with a gradual increase in time, we studied the infection phenotypes by microscopy on the leaves of Col-0, rss1 rss1–1, and rss1–2 using trypan blue in the whole leaf (Fig. 3D). Among all, rss1 showed an enhanced mycelial mass of RS and maximum lateral branching marked by irregular-shaped dark blue regions on the epidermal cell surface, shown by infection on the whole leaf (left panel) and microscopic image (right panel) at 1 dpi. At 2 dpi and 3 dpi, RS continued to proliferate in rss1 with profuse mycelial branching, extensive colonization, and the formation of infection cushions (Fig. 3D). rss1–1 and rss1–2 indistinctly showed similar infection severity like rss1 whereas Col-0 only showed entangled, right-angled branching of mycelia entrenched on the leaf surface with increasing time points. The image represents one of the representative experiments from each ecotype, with similar results. Based on these microscopic images, the relative intensity of trypan blue was quantified, showing higher intensity in rss1 than rss1–1 and rss1–2 which was normalized with the intensity of Col-0 (Fig. 3E).

To monitor the induced cell death after infection, the percentage of released electrolytes was measured. The increase in cell death count was significantly higher in rss1 than rss1–1 and rss1–2 as compared to Col-0. rss1 showed an increase of ~70% ion leakage at 3 dpi (Fig. 3F). Our observation indicates that RS infection in rss1 was found to be increasing in a spatiotemporal manner. To further visualize the infection in rss1, we checked the micrograph with z-stacking in a confocal microscope (staining by calcfluor White) and found the surface to in-depth spread and colonization of the entangled mycelia at 3 dpi (Figs. 3G, S3). The infection cushion development was barely visible in Col-0, whereas there were enormous infection cushions that are evident in the rss1 and rss1–1. This suggests that the significant breach in immunity is due to point mutation in the IMPA2 and its knockout in Arabidopsis respectively.

3.4. Impaired IMPA2 and its knockouts show accumulation of RS-induced reactive oxygen species and callose deposition

Production of hydrogen reactive oxygen species and accumulation of callose at the site of successful infection in the plant are the early defense responses to biotic stress (Bolwell and Daoudi, 2009; Ellinger and Voigt, 2014). We investigated the pattern of H2O2 accumulation from Arabidopsis in rss1 at 1 dpi, 2 dpi, and 3 dpi. Microscopy images depict higher H2O2 accumulation in rss1 than the Col-0 with increasing time points (Fig. 4A). Furthermore, the T-DNA insertion mutants rss1–1 and rss1–2 also showed similar phenotypes like rss1 leaves. The relative intensity of DAB was also quantified digitally which showed a significant difference among Col-0 and respective rss mutants (Fig. 4B). This result was further substantiated by using H2DCFDA in live infected tissues, which upon reaction with ROS produces a highly fluorescent product i.e., 2′, 7′-dichlorofluorescein. rss1 and T-DNA insertion lines showed maximum localization and accumulation of ROS which were indistinctly accumulated in the Col-0 cells at 30 hpi (Fig. 4C). Relative fluorescence intensity of H2DCFDA showed a significant difference among Col-0 and mutants (Fig. 4D). A higher accumulation of superoxide ions, O2•−, another important component of ROS, was observed in the infected leaves of rss1, rss1–1, and rss1–2 at 30 hpi using NBT stain (Fig. 4E). This correlation amongst the microscopic symptoms augmented levels of H2O2 and O2•− accumulation in rss1 and SALK mutants suggests that RSS1 could be involved in negatively regulating hypersensitive response and compromised penetration of RS.

Callose, a (1,3)-β-glucan cell wall polymer, is a common constituent of papillae formation which marks the entry sites of a pathogen (Aist, 1976). Papillae act as a physical barrier that restrict pathogen colonization (Stone and Clarke, 1992). An intensified noticeable callose deposition was observed at the fungal infection sites of inoculated
mutant leaves as an attempted entry by RS when assessed with the WT at 3 dpi (Fig. 4F). An increase in callose deposition was observed in rss1 and rss1–1 leaves upon infection which however is inhibited in WT, which may be preventing further entry of the pathogen. This reveals that the mutant can still recognize the infection and react competently. Relative expression of GSL5, a pathogen-induced callose biosynthesis marker (Enns et al., 2005), was also measured. Noticeable enhancement in the GSL5 expression was observed in rss1 followed by rss2 (Fig. 4G). Taken together, our results indicate that RSS1 is actively involved in both pre-and post-invasive defense response, as a mutation in this gene leads to elicited accumulation of ROS and papillae formation. Furthermore, rapidly activated cellular defense response without any hypersensitive response to infection by RS in Col-0 indicates its IMPA2-mediated NHR.

3.5. RS infection in rss1 affects photosynthetic efficiency and chlorophyll biosynthesis

Chloroplast biosynthesis plays a major role during plant immunity where mesophyll chloroplasts are the common regulator of photosynthesis (Trieda and Takano, 2021; Serrano et al., 2016). Interestingly, microscopic autofluorescence images and the observations of RS challenged rss1 leaves at 2 dpi displayed a reduced amount and their distribution of photosynthetic pigment. The dynamics of pigments in the mesophyll of infected leaves of Col-0 showed higher chloroplast autofluorescence with unbounded clustering within the cells. On the
contrary, Col-0 and rss1 showed indistinctive chloroplast autofluorescence in the mock leaves (Fig. 5A). Since the effect of RS infection in rss1 is seen on its biosynthesis, we quantified chlorophyll (chl) pigments. RS infection led to a maximum decrease in the content of chl a (Fig. 5B), chl b (Fig. 5C), and total chl (Fig. 5D) as the time progressed as compared to Col-0. The T-DNA insertion lines also showed a lower amount of chlorophyll pigments; however, the pattern was similar for chl b and total chl but rss1 showed a drastic reduction in chl a, compared
to Col-0. This data indicates that in the absence of RSS1 the released effector or toxins from RS during infection might act on the chloroplastic proteins deleteriously. This might lead to reduced chlorophyll synthesis, consequently directing to impede in photosynthesis and associated defenses resulting from this center.

3.6. Mutation in IMPA2 affects rss1 seedling phenotype with developmental defects

We aimed to assess developmental processes due to mutation in RSS1 and performed a seed germination assay of Col-0 and rss1 under similar illumination, temperature, and humidity conditions. The germination rate of the mutant was slower than that of the Col-0 on the 10th day from seed sowing (Fig. S9Ai, ii). The seeds of Col-0 showed a higher germination rate of 52.6% whereas it was 84.4% in the rss1 (Fig. S9b). The 15th day old Col-0 and rss1 mutants showed a significant reduction in the root length in rss1 was seen as compared to the Col-0 (Fig. S9Aiii). The primary root length of Col-0 and mutant were recorded in the 15th-day-old seedlings and found to be 2.38 cm and 0.66 cm respectively (Fig. S9C). Further, the number of lateral roots in Col-0 was more than that of mutant (Fig. S9D). Additionally, Col-0 and mutant rosette leaves didn’t show any morphological difference except that Col-0 rosette leaves have extended petioles and long-oval-shaped leaves (Fig. S9Aiv). The number of rosette leaves on the 10th, 20th, and 30th day were significantly less in rss1 i.e., around 2, 7, and 14 respectively which was around 4, 15, and 24 leaves in the Col-0 (Fig. S9E). Several fully developed siliques and several seeds produced per siliques also altered in rss1 (Fig. S9F, G). Although there was little morphological difference in the silique phenotype of Col-0 and rss1 (Fig. S9Av), the length of silique did not show any significant difference in length (Fig. S9H). Also, the induction of flowering and length of the plant was deferred in rss1 (Fig. S9Avi,6i).

3.7. RSS1 provides salicylic acid-mediated defense in response to RS-infection

To elucidate the molecular mechanism involved during RS pathogenesis, well-characterized subsets of defense-related signaling pathway component reporter genes were inspected in Col-0, rss1, and rss1–1 with an increase in time course by RT-qPCR. We observed significantly induced expression of PDF1.2a upon infection that kept on increasing up to 3 dpi in rss1, rss1–1, and rice (Fig. 6 A). However, Col-0 showed a marginal increase in PDF1.2a expression. As a measure of defense to the invading pathogen, Col-0 showed a gradual increase in expression of ~13-fold at 3 dpi of PR1 (SA response and immunity) and mutants including rice had a decrease in expression concerning time. This may be resulting from compromise in immunity and worked as an antagonistic marker expression (PDF1.2) as seen for the jasmonic acid. Surprisingly, the response of PR1 expression to infection showed a steep increase at 1 dpi in rss1, rss1–1, and rice (~18, ~14, and ~15 fold respectively) (Fig. 6B) whereas only 2.5-fold overexpression was seen in Col-0. The spiked enhancement in PR1 expression in mutants and rice at 1 dpi upon RS infection and its downregulation with an increase of PDF1.2 expression suggests that the necrotrophic phase of the pathogen is...
activated upon subduing early defense. Furthermore, enhancement in PR1 expression correlating to the studied disease resistance phenotype up to 3dpi in Col-0 marks that there is an active SA mediated immunity against RS infection. We also investigated the expression pattern of the gene ICS1 that is required for SA biosynthesis and observed that its expression at 3dpi in Col-0 was ~6 fold whereas, in the rss1 and rss1–1, it was only <2- and <3-fold respectively. This result further confirms the SA mediated active defense in Col-0 against RS governed by IMPA2 (Fig. 6C). To check the elevation in HR to RS challenge, the expression pattern of AtNHL10 was studied and we found that ~60-fold increased expression in Col-0 at 3-dpi but none in rss1 and rss1–1 (Fig. 6D) indicating the SA signaling pathway involvement in immunity in Arabidopsis (Zheng et al., 2004).

We reasoned that the infected cells in rss1 and rss1–1 might be leading to autophagic cell death and determined the AtATG8a expression pattern during the infection. We did not observe any expression of ATG8 in Col-0 upon infection which also correlates with the infection phenotype where no infection cushions or tissue damage was observed. This suggests that induction of autophagic cell death in Col-0 is not seen, possibly by the active function of IMPA2 (Fig. 6E). On the contrary, rss1 and rss1–1 showed enhanced expression of ATG8 with an indication that autophagic cell death induced by RS signifying successful empowerment of its necrotrophic behavior (Wang et al., 2020). We wanted to investigate if IMPA2 expression is also regulated by RS infection and observed gradual downregulation in its expression in Col-0 signifying its effect on the active participation during immunity. On the other hand, the water control samples of rss1 showed a decrease in expression which even showed further decreased expression as time progressed and weaker expression in the rss1–1 (Fig. 6F). We did not observe any expression of ATG6 and SEN1 signifying the absence of related pathways for defense or no involvement of them in RS infection.

3.8. A homolog of IMPA2 and nuclear RAN1 functions together in triggering immunity

The homology search analysis depicted IMPA1 as the close homolog with P conserved like IMPA2 (Fig. 7A). To access the plausible involvement of IMPA1, the screening of a homozygous T-DNA insertion line in IMPA1 (SALK_082616) was performed. It showed a similar susceptible phenotype that indicates both IMPA2 and IMPA1 independently are involved in providing resistance against RS (Fig. 7). Although rice ortholog (Os01g14950) has conserved proline at 60th position, it is
working as a host to RS, whereas Col-0 with its wild type IMPA2 is nonhost. Further, to ascertain the functionality of the ternary complex made from the binding of importin α and importin β that transports inside the nucleus and further destabilization of the complex via GTP bound RAN protein inside nucleoplasm (Lüdke et al., 2021), we chose to study the involvement of \( \text{RAN1} \) (At5g20010). Surprisingly, we found that the knockout mutant of \( \text{RAN1} \) also showed a similar susceptibility response towards infection by RS, which however was not seen in the case of another knockout mutant of \( \text{ABC1} \) (SALK_045739) (Fig. 7B,C).

The subcellular localization of IMPA2 denotes higher expression in cytosol followed by the nucleus and is lower in the cell wall, plasma membrane, mitochondria, and Golgi but no expression is seen in the plastid (bar.utoronto.ca/cell_efp). The above result suggests that IMPA2, IMPA1, and \( \text{RAN1} \) function together and activate immunity against RS (Fig. 7D).

4. Discussion

4.1. IMPA2 impaired at \( \text{P}^65 \text{S} \) in Arabidopsis shows breached immunity to RS

Although there has been no report on the potential rice resistant germplasm, natural nonhost variants that diverged across evolutionary timescale provide resistance by curtailing the early stages of growth in Arabidopsis against RS (Parween et al., 2021). Here we have applied forward genetics and identified IMPA2 (a karyopherin), an NHR gene that provides resistance mediated by SA biosynthetic pathway activation. The identified NHR gene, \( \text{At4g16143} \) encodes an IMPA2 which is
ubiquitously expressed bearing nuclear localizing signal, NLS. There are 9 paralogs of importin in Arabidopsis with 10 ARM repeat domains whereas six orthologs in rice (Table S7). The distinct functionalities of a specific group of importins in Arabidopsis and rice indicate that the individual importin mediates unique modalities of nuclear import (Quensel et al., 2004). A compromise in the signaling of immunity upon mutation in IMPA3 has been reported to function upon interaction with mutated SNCl (TIR-NBS-LRR-type) (Palma et al., 2005). Arabidopsis IMPA3 mutation leads to nucleocytoplasmic SNC indicating the requirement of this protein for import of specific signaling components to the nucleus (Zhu et al., 2010). Specific interaction and their import to the nucleus is seen in the case of effectors Nuk6 and Nuk7 but not Nuk12 of P. infestans upon silencing of N. benthamiana IMPORTIN ALPHA 1 and IMPORTIN ALPHA 2 (Kanneganti et al., 2007). Arabidopsis IMPORTIN ALPHA 4 has a significant contribution as a requirement in T-complex transport, VirE2 and VirD2 import to nucleus towards the successful transformation by Agrobacterium (Bhattacharjee et al., 2008). A wide array of effectors have been reported to localize to nuclei that indicate their sequesteriation via import or passive diffusion (Caillaul et al., 2012; Deslandes and Rivas, 2011). Hetero trimerization of importin alpha loaded with target protein via binding to the armadillo domains with importin beta 1 ferry to the nucleus and with the help of Ran GTP it dissociates and is recycled back to the cytoplasm (Lüdke et al., 2021; Mattaj and Englmeier, 1998; Miyamoto et al., 2016). Our results of specific susceptible disease phenotype in the EMS generated rss1 with the truncated 65s in the IBB domain which is autoregulatory and the rss1–1 where no expression of IMPA2 is seen (Fig 2F). This may indicate the target-specific binding of the protein to the cargo peptides for downstream activation of the defense signaling cascade. In addition to the function of IMPA2, IMPA1 also has a pivotal role in defending the pathogen (Fig. 7). Also, IMPA2 interacts with RANBP1A (https://genemania.org/) and RANBP1A interacts with RAN1, RAN2, and RAN3 (Haizel et al., 1997). RAN1 also functions in defense signaling which is confirmed from the knockout phenotype study against RS (Fig. 7). Whether there is any canonical NLS signal with the effector(s) or specific group of putative toxins of RS that are secreted during the onset of infection and what are the molecular interacting partners in defining the resistance in Col-0 needs further studies.

4.2. Mutation in RSS1 activates early defense in Arabidopsis in response to RS and negatively regulate chlorophyll biosynthesis

The degree of infection cushion formation and cell death in the mutants was comparable to rice. The rapid accumulation of H2O2, O2•− and deposition of callose at the infection sites are the major hallmark events of cellular defense response in plant cells (Conrath et al., 2001) which play pivotal roles in defense against necrotrophic fungal intruders (Lopez-Cruz et al., 2017; Su udi et al., 2011). We studied the accumulation of H2O2, O2•− and deposition of callose in NHR to RS infection. The results of dramatic difference in the rss1, rss1–1, and rss1–2 as compared to Col-0 suggests that cellular defense is activated in response to infection and entry of the fungal mycelia (Fig. 3). Intriguingly, the cell death did not curtail the further growth of the pathogen and the infection still aggravated suggests that the cell death during HR is a consequence of the cessation of further growth which is found in the compatible rss1 (Salient-Kurti, 2019). Epidermal chloroplast is the motive organelles that position immune components and fend off pathogen intruders (Irieda and Takano, 2021). The rss1 was breached with pre-penetration resistance and the mycelia could establish itself inside the epidermis. Possibly, the effectors target to reduce the Chlorophyll biogenesis and the infected leaves showed reduced content of the photosynthetic pigments (Fig. 5). Furthermore, the developmental control of the EMS mutant indicates the possible role of IMPA2 (Fig. 6).

4.3. SA activates immunity against RS and impa2 shows autophagic cell death

Activation of the signature defense pathway genes in biotic stress is reported to respond to pathogen entry to the tissue (Chan and Zimmerli, 2019). We also aimed to investigate the responses underlying the responses to RS infection and found that the PR1 expression was induced in Col-0 which may be because early defense responses with no establishment of the fungus are provided by SA mediated defense (Fig. 7). However, the mutants showed the opposite trend with a huge increase of PR1 expression at the onset of infection and thereby a decrease at 3 dpi. The SA biosynthesis marker expression was also elevated in response to RS infection confirming the SA mediated defense response in Col-0 at the early hours of immunity. Interestingly, the HR marker gene NHL10 was found to elevate ~60 fold in Col-0 even at 3 dpi but none at 3 dpi confirming the fact that the cell death in mutants is pathogen-induced and Col-0 showed HR and did not allow the formation of infection cushions. The wheat pathogen, Parastagonospora nodorum, a necrotroph induces programmed cell death by secreting the host-specific toxins to derive nutrition and do not block further invasion (Lorang, 2019). Our results on the induction of the ATG8 in rss1 and rss1–1 but null in the Col-0 correlate with the RS-induced autophagic cell death with plausible toxins/effectors as seen in the photomicrographs (Fig. 3, 4, and 7). However, further studies are required/ to confirm the autophagy regulation conferred by IMPA2. The downregulation in the expression of the IMPA2 in RS challenged leaves indicates that it positively regulates the immunity against the establishment and subverting the gene expression helps it in establishing the mycelial mass after 3 dpi in the nonhost, Col-0. Furthermore, the minor expression of the gene does not perform any function in activating HR and leads to death from which the fungus derives nutrition. However, which effectors or toxins are involved in infection and what is the path of nuclear programming in immunity in Arabidopsis against RS remains to be investigated.

In summary, the Arabidopsis IMPA2 has a significant contribution towards activating HR against RS. The functionality of IMPA2 plausibly might be in the transport of the cargo effectors that prove vital in immunity against RS in plants. This provides evidence on the first clue for the involvement of an NHR gene, IMPA2 against RS infection. This would be possibly helpful for use to genetically engineer elite rice cultivars with improved immunity that is durable, broad-spectrum, and lead a sustainable environment.

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Data availability

Data will be available upon request.

Supplementary information

Table S1. Chi-square tests for Mendelian 3:1 segregation of the Col-0 (resistant) to rss1 (susceptible).

Table S2. Fine mapping of RSS1 using individual F2:3 susceptible samples on Chromosome 4.

Table S3. List of putative RSS1 genes identified from the NGM analysis pipeline upon alignment onto Col-0 reference sequence.

Table S4. Details of the SALK T-DNA insertion lines used for confirmation of the mutation.

Table S5. List of SSLP primers used for qPCR.

Table S6. List of primers used for RT-qPCR.

Table S7. Percentage of similarity index of IMPA2 with 9 paralogs in Arabidopsis and six orthologs in rice.

Fig. S1. Phenotype and screening of EMS mutated M3 plants.
Fig. S2. Genotype confirmation of F1 and phenotype screening of F2 plants from 0.3% EMS mutated plants.

Fig. S3. Micrographs of Calcofluor White stained surface mycelia of RS.

Fig. S4. Chromosomal mapping and polymorphism showed by SSLP markers in Chromosome 1.

Fig. S5. Chromosomal mapping and polymorphism showed by SSLP markers in Chromosome 2.

Fig. S6. Chromosomal mapping and polymorphism showed by SSLP markers in Chromosome 3.

Fig. S7. Chromosomal mapping and polymorphism showed by SSLP markers in Chromosome 4.

Fig. S8. Chromosomal mapping and polymorphism showed by SSLP markers in Chromosome 5.

Fig. S9. Comparison of the phenotypic variations between wild type and EMS generated mutant explained impaired development in rs1.

CRediT authorship contribution statement

Daraksha Parween: Visualization, Investigation, Formal analysis. Writing – original draft. Binod Bihari Sahu: Conceptualization, Supervision, Formal analysis, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Daraksha Parween reports financial support was provided by Science and Technology department, Government of Odisha. Binod Bihari Sahu reports financial support was provided by Science and Technology Department, Government of Odisha.

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Supplementary materials

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