



Letter

Generation of *Brassica napus* with enhanced *Sclerotinia sclerotiorum* resistance through CRISPR/Cas9-mediated inhibition of the PROTEOLYSIS6 N-degron pathway

Introduction

Sclerotinia sclerotiorum is a devastating necrotrophic fungal pathogen with an exceptionally broad host range, infecting over 700 plant species. Brassicaceae species are particularly affected by this pathogen, including economically important species such as *Brassica napus* and various vegetable plants like *Brassica rapa* L. ssp. *pekinensis* (Liu *et al.*, 2025). This pathogen causes significant reductions in both yield and quality, and to date, no fully resistant germplasm has been identified. Consequently, the identification and genetic manipulation of disease-resistant genes represent a pivotal strategy for enhancing host resistance against *S. sclerotiorum*. Accumulating evidence indicates that biotic stresses, including those by necrotrophic pathogens such as *Botrytis cinerea* (Valeri *et al.*, 2021) and in the clubroot agent *Plasmodiophora brassicae* (Gravot *et al.*, 2016) and bacterium *Agrobacterium tumefaciens* (Kerpen *et al.*, 2019), induce hypoxic stress in plant tissues. Intriguingly, studies in *Arabidopsis thaliana* demonstrate that hypoxia may compromise plant immunity by suppressing PAMP-triggered immunity (Mooney *et al.*, 2024), highlighting the potential role of oxygen (O₂) deprivation as a common aspect during different biotic stresses.

In this study, we applied multiple approaches to demonstrate that infection by *S. sclerotiorum* induces hypoxic stress in host plants. Building on this finding, we used CRISPR/Cas9-mediated knockout of *BnaPROTEOLYSIS (PRT)6* to disrupt the low-oxygen-associated N-degron pathway, thereby conferring strong resistance to *S. sclerotiorum* in *B. napus*.

Results and Discussion

Sclerotinia sclerotiorum infection establishes hypoxic conditions within plant cells

To investigate whether *S. sclerotiorum* infection induces hypoxic stress in host plants, a comparative RNA-Seq analysis was conducted on *B. napus*. Plants were subjected to either 3-h

submergence, which has been used as a well-established hypoxia treatment, or 12-h *S. sclerotiorum* inoculation. Transcriptomic analysis revealed that 34 out of 43 (79%) hypoxia-responsive marker genes (Mustroph *et al.*, 2009) exhibited nearly identical upregulation patterns under both conditions, strongly suggesting that *S. sclerotiorum* infection triggers a strong hypoxia response (Fig. 1a; Supporting Information Fig. S1). This finding was further substantiated using a planar O₂ sensor system, which comprises an O₂-sensitive optical foil integrated with a high-definition detection microscope unit for detailed spatial measurement of O₂ tension (Figs 1b, S2). The measured ratio shows a negative correlation with the relative O₂ concentration. Real-time monitoring over a 500-s period demonstrated that O₂ concentrations in infected tissues were consistently and significantly lower than in adjacent uninfected regions, establishing a clear negative correlation between pathogen infection and local O₂ availability (Fig. 1c; Video S1). To accurately quantify O₂ concentration at the cellular level, we utilized high-resolution microsensors technology to measure O₂ concentrations in *S. sclerotiorum* infected and uninfected abaxial lamina (Figs 1d, S2). The O₂ tension in uninfected leaf tissues was c. 20 kPa, whereas in infected tissues it decreased to c. 5 kPa (Fig. 1e). These measurements confirmed a statistically significant reduction in O₂ availability in pathogen-infected leaves compared to uninfected controls.

The PLANT CYSTEINE OXIDASE (PCO) N-degron pathway is the only known mechanism mediating cellular sensing and response to hypoxia in plants (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Central to this pathway are the Group VII Ethylene Response Factor (ERFVII) transcription factors (in *A. thaliana* RAP2.12, RAP2.2, RAP2.3, HRE1, and HRE2), which function as physiological substrates for oxygen-dependent N-degron-mediated proteasomal degradation catalyzed by candidate E3 ubiquitin ligase PROTEOLYSIS 6 (PRT6) (Zubrycka *et al.*, 2023) (Fig. 1f). Transduction of oxygen sensing is dependent on Cys-2 of ERFVIIs, in the presence of oxygen this residue is oxidized resulting in protein degradation, whereas in hypoxia the protein is stable (Manrique Gil *et al.*, 2025). This residue is part of a larger amino-terminal conserved region in ERFVIIs (Nakano *et al.*, 2006). We identified the conserved amino-terminal residues of BnaERFVIIIs (Fig. 1g–i) and generated GUS reporter proteins beginning with the ERFVII sequences MCGGAI/VI (Fig. 1j) to investigate the influence of *S. sclerotiorum* inoculation on stabilization through these sequences. Whereas little GUS activity was observed in mock leaves with the wild-type sequences, substitution of Cys-2 with Ala (a residue that does not act as a destabilizing residue in the N-degron pathway) resulted in constitutive activity of GUS regardless of infection. Following infection with *S. sclerotiorum*, GUS reporter activity using both wild-type sequences greatly increased (Fig. 1k). These results suggest that hypoxic conditions induced by *S. sclerotiorum* infection promote protein stabilization through Cys-2,

thereby providing a mechanistic explanation for how reduced oxygen availability enhances protein stability and function.

To further investigate the functional role of this pathway in plant resistance to *S. sclerotiorum*, we performed *S. sclerotiorum* inoculation assays using *A. thaliana* mutants defective in either all five ERFVII transcription factors (pentuple mutant *erfVII* (Abbas *et al.*, 2015)) or the N-recognin PRT6 (*prt6-1*) (Fig. 11). Quantitative analysis revealed that leaves of *erfVII* mutants developed significantly larger lesion areas compared to wild-type plants following *S. sclerotiorum* inoculation (Fig. 1m), indicating that ERFVII transcription factors play an important role in defense against this pathogen. By contrast, the *prt6-1* mutant (in which substrates are stable) exhibited enhanced resistance, with lesion areas reduced by 16.7% relative to controls. These data demonstrate that the PRT6 N-degron pathway negatively regulates plant defense against *S. sclerotiorum*, likely through its control of ERFVII protein stability. Our findings suggest that targeted genetic disruption of PRT6 function represents a promising strategy for engineering *S. sclerotiorum* resistance in plants.

Genetic knockout of PRT6 significantly enhances host resistance against *S. sclerotiorum* in *B. napus*

Brassica napus is not only a crop of major global economic importance but is also widely regarded as a model system for studying polyploidy within the Brassicaceae family, and specifically in the genus *Brassica*. In *B. napus*, we identified six PRT6 homoeologs through genome-wide analysis, with four homoeologs (BnaA02.PRT6 (A), BnaC02.PRT6 (B), BnaA10.PRT6 (C), and BnaC09.PRT6 (D)) exhibiting relatively high expression levels across multiple tissues (Fig. S3). Using CRISPR/Cas9 genome editing, we designed four specific guide RNAs targeting conserved functional domains in these homoeologs (Fig. 2a,b). Among the 13 positive transgenic T₀ plants, five (38.5%) exhibited successful genome editing events. These included two single mutants (#2 and #6), one double mutant (#30), one triple mutant (#29), and one quadruple mutant (#23) (Fig. 2c). All mutations were heterozygous in the T₀ generation. Following two generations of segregation and genotyping, we isolated plants carrying stable homozygous and multiplex edited alleles, including single mutant *bnac02.prt6* (bb), double mutant *bnac02.prt6 bnac09.prt6* (bbdd), triple mutant *bnac02.prt6 bnaa10.prt6 bnac09.prt6* (aabbdd), and quadruple mutant *bnaa02.prt6 bnac02.prt6 bnaa10.prt6 bnac09.prt6* (aabbccdd) genotypes. These were verified via PCR amplification followed by Sanger sequencing using allele-specific primers (Fig. S4). Previously, it was shown that in the *Arabidopsis* *prt6* mutant, hypoxia response genes are constitutively upregulated (Gibbs *et al.*, 2011). Here, we also found strong upregulation of hypoxia key response genes (including *ADH1* and *PDC2*) in the *bnaprt6* (aabbccdd) mutant compared to wild-type plants (Fig. S5).

Pathogenicity assays using *S. sclerotiorum* isolates revealed a striking gene dosage effect, with disease resistance strongly correlating with the number of disrupted PRT6 copies (Fig. 2d,e). Quantitative analysis showed that while single mutants (bb) exhibited only a modest, non-significant reduction of 1.4% in lesion area, higher-order mutants demonstrated progressively

greater resistance. Double mutants (bbdd) showed 11.7% reduction, triple mutants (bbccdd) 27.3%, and quadruple mutants (aabbccdd) 29.1% compared to wild-type controls (Fig. 2e). Comprehensive evaluation of key agronomic traits in field-grown plants revealed that these *PRT6* null lines exhibited no significant alterations in plant height, branch number, main inflorescence silique number, or thousand-seed weight compared to wild-type plants (Fig. 2f). In addition, no significant impact on rapeseed quality was observed (Table S1). These findings suggest that targeted disruption of *BnaPRT6* genes can enhance *S. sclerotiorum* resistance without compromising essential agronomic performance, a crucial consideration for crop improvement strategies. To facilitate molecular marker-assisted selection, allele-specific markers were developed to accurately distinguish among wild-type, heterozygous, and homozygous mutant genotypes of four *BnaPRT6* homoeologs (Figs 2g, S6). Future studies should evaluate the performance of these mutants under field conditions, with particular emphasis on resistance of the stem and other key organs to *S. sclerotiorum*.

In summary, our study demonstrates that *S. sclerotiorum* infection induces hypoxic conditions in plant tissues. Furthermore, the *A. thaliana* *prt6* mutant exhibits enhanced resistance to this fungal pathogen, as has been shown for other biotic stresses (Vicente *et al.*, 2019; Valeri *et al.*, 2021), indicating an important role for the PRT6 N-degron pathway in resistance to this fungus. Based on these findings, we successfully generated *Sclerotinia*-resistant *B. napus* by disrupting the N-degron pathway through targeted knockout of *BnaPRT6* using CRISPR/Cas9 technology (Fig. 2h). The evolutionary conservation of the PRT6 N-degron pathway across higher plants, combined with our demonstration of its role in *S. sclerotiorum* resistance, highlights its potential as a broad-spectrum target against *S. sclerotiorum*. Notably, PRT6 orthologs show high sequence conservation across the Brassicaceae family (Table S2), suggesting our findings will be translatable to other economically important species vulnerable to *S. sclerotiorum*. Beyond the findings presented in this study, removal of PRT6 function has previously been shown to confer tolerance to abiotic stresses such as drought, salinity, and waterlogging in *Arabidopsis* and barley (Mendiondo *et al.*, 2016; Vicente *et al.*, 2019). This raises the possibility that the genetic resources generated here may underpin broad-spectrum tolerance to both biotic and abiotic environmental challenges, an avenue that warrants further investigation.

Materials and Methods

Plant material and growth conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana*, originally obtained from the Nottingham Arabidopsis Stock Centre (NASC), was used as the wild-type background for this study. *Arabidopsis* mutant seeds *erfVII* and *prt6-1* utilized in this study were previously developed (Gibbs *et al.*, 2011; Weits *et al.*, 2014; Abbas *et al.*, 2015).

Brassica napus cultivar J9712 was used as explant for oilseed rape hypocotyl transformation. This was originally provided by Prof.

Yongming Zhou (Huazhong Agricultural University, Wuhan, Hubei, China). The *BnaPRT6* genome sequence, the gene coding sequence (CDS), and the protein sequence were acquired from an online database (<https://yanglab.hzau.edu.cn/BnIR>). Four sequence-specific single guide RNAs (sgRNAs) were designed using the online tool CRISPR-P 2.0 (<http://cbi.hzau.edu.cn/CRISPR2/>). These

sgRNAs were incorporated into a CRISPR/Cas9 construct using the pRGE32 multiplex genome targeting vector, generously provided by Prof. Kabin Xie (Huazhong Agricultural University, Wuhan, Hubei, China). The genome-edited plants were obtained through *Agrobacterium*-mediated transformation. Positive transformants harboring T-DNA insertions were initially screened by PCR

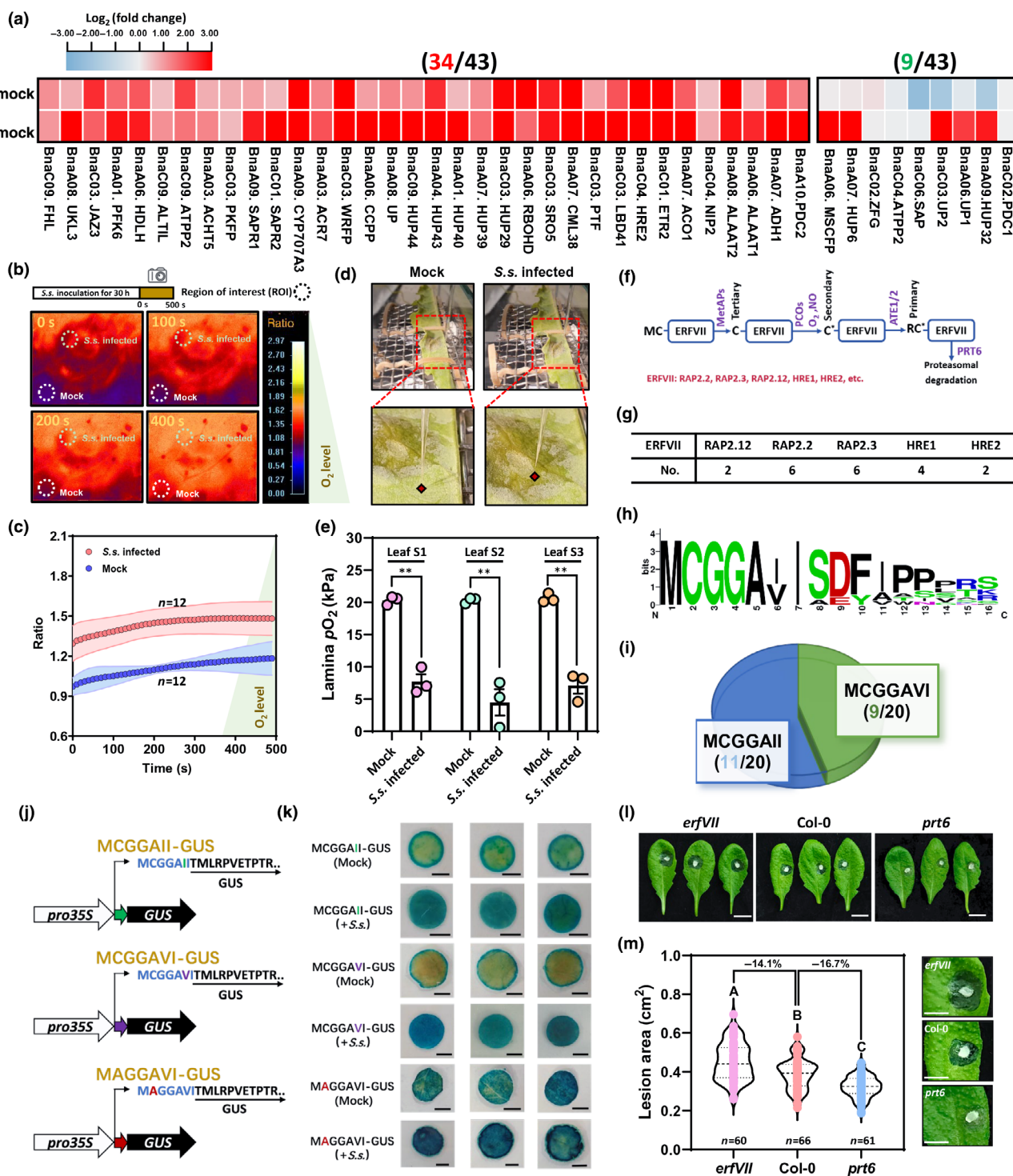


Fig. 1 *Sclerotinia sclerotiorum* (S.s.) infection establishes cellular hypoxia in *Brassica napus*. (a) Transcriptomic analysis of hypoxia-induced marker genes following either 3 h submergence (Sub.) treatment or 12 h S.s. inoculation. The red and green numbers represent the number of genes with consistent and divergent expression trends, respectively, in response to both submergence and S.s. inoculation. (b) Representative images visualizing O₂ concentration in mock-treated and S.s.-infected areas with planar O₂ sensor. (c) Quantification of relative O₂ levels in mock-treated and S.s.-infected areas every 10 sec with planar O₂ sensor. Green triangle indicates a negative correlation between the ratio and O₂ level. Data are presented as mean $\pm$ SE ($n = 12$) from four independent experiments. (d) Images from microsensor detection showing O₂ levels in mock-treated and S.s.-infected areas. (e) O₂ measurements in mock-treated and S.s.-infected areas. Data are presented as mean $\pm$ SE from three independent experiments ($n = 3$, **, $P < 0.01$, Student t -test). (f) Schematic model of the PCO N-degron pathway in Arabidopsis highlighting the four key enzymes required for ERFVII degradation and the position of oxygen sensing by PCOs, C* represents oxidized Cys. (g) Number of ERFVII transcription factors (RAP2.12, RAP2.2, RAP2.3, HRE1, HRE2) in *B. napus* Zhongshuang11 (ZS11). (h) Conservation analysis of the first 16 amino acids of the ERFVII transcription factors. The logo plot was generated using WebLogo (<http://weblogo.berkeley.edu/>). (i) The first 7 amino acids can be classified into two groups: MCGGAI (11/20) and MCGGAVI (9/20). (j) Schematic diagram of the vector constructs. MCGGAI and MCGGAVI were individually fused to the GUS reporter gene and used for genetic transformation of *B. napus* cultivar J9712. The construct with MAGGAVI fused to GUS served as a positive control. (k) GUS staining results following inoculation with or without S.s. Bars, 5 mm. (l) Representative images of S.s.-inoculated leaves from Col-0 accession (wild-type), *erfVII* and *prt6-1* mutant plants. Bars, 1 cm. The enlarged images below show the difference in lesion area between the genotypes. Bars, 5 mm. (m) Quantification of lesion area on inoculated leaves. Data are presented as mean $\pm$ SE with three independent biological replicates ($n = 60$ –66, ANOVA with Tukey's test, $P < 0.01$). Quartiles and the median are shown by dotted and dashed lines, respectively.

amplification. Homozygous *BnaPRT6* knockout mutants were subsequently confirmed using PCR followed by Sanger sequencing. Allele-specific molecular markers were designed according to the precise nucleotide insertion sites in each homoeologous gene (Lin *et al.*, 2024). After obtaining the mutant lines, specific primers were designed to detect potential off-target mutations in the non-targeted PRT6 homeologs (*BnaA03.PRT6* and *BnaC03.PRT6*) (Table S3).

Arabidopsis seeds were germinated on vertical plates containing an agar medium, which consisted of 1/2 Murashige & Skoog basal salt mixture (Duchefa, the Netherlands), 0.8% plant agar, and 1% sucrose (Sigma-Aldrich, Darmstadt, Germany). The seeds were stratified at 4°C in the dark for 48 h before being transferred to a growth environment at 22°C, with a 16 h : 8 h light : dark photoperiod. After 7 d, the seedlings were transplanted into pots with soil for continued growth. Similarly, *B. napus* plants were cultivated in a climate-controlled chamber under the same light conditions (16 h : 8 h light : dark photoperiod) at 22°C, with a maintained humidity level of 65%. To assess susceptibility to *Sclerotinia sclerotiorum* (Lib.) de Bary, wild-type J9712 and mutant plants were cultivated in the field for *c.* 5 months before leaf collection.

Bioinformatic analysis

The protein sequences of ERFVII transcription factors (RAP2.12, RAP2.2, RAP2.3, HRE1, and HRE2) in *B. napus* Zhongshuang11 (ZS11) were retrieved from the online database BnIR (<https://yanglab.hzau.edu.cn/BnIR>). The first 16 N-terminal amino acids of each ERFVII protein were extracted for conservation analysis. A sequence logo was generated using WebLogo (<http://weblogo.berkeley.edu/>), and a pie chart was prepared with Microsoft Excel.

A phylogenetic tree was constructed based on the amino acid sequences of six *BnaPRT6* homeologs obtained from the BnTIR database (<http://yanglab.hzau.edu.cn/BnTIR>). The tree was generated in MEGA11 using the neighbor-joining method with 1000 bootstrap replicates. Expression patterns of the six *BnaPRT6* homeologs across different plant tissues and organs were also

retrieved from the BnTIR database. Information on PRT6 orthologs in plant species of the Brassicaceae family was found in the Brassicaceae Database (BRAD) (<http://brassicadb.cn>).

β -Glucuronidase (GUS) histochemical staining

The coding sequences of MCGGAI-GUS, MCGGAVI-GUS, and MAGGAVI-GUS were cloned into the binary vector pMDC83 and subsequently introduced into the *B. napus* line J9712 via *Agrobacterium*-mediated transformation. Putative transgenic plants containing the T-DNA insertions were first identified by PCR screening. Homozygous T₂ lines, derived from these primary transformants, were then used for GUS staining analysis. Detached leaves from transgenic plants were inoculated with a 7-mm-diameter *S. sclerotiorum*-colonized agar plug. Leaves were collected at 16 h post-inoculation, allowing sufficient hyphal colonization while retaining structural integrity for subsequent analyses. As a control, mock inoculation used a sterile agar plug of identical size. A 10-mm-diameter leaf disc was then excised from the inoculation site for histochemical GUS staining. Histochemical GUS staining was conducted according to Jefferson *et al.* (1987). Briefly, leaf tissues were incubated overnight at 37°C in staining solution containing 0.5 mg ml⁻¹ X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% Triton X-100, and 10 mM sodium phosphate buffer (pH = 7.2). Subsequently, samples were destained in 75% (v/v) ethanol and imaged under a stereomicroscope (Leica EZ4W, Bensheim, Germany) equipped with a digital camera.

Quantitative real-time PCR

Brassica napus seedlings grown on Whatman paper were harvested 6 d post-germination for total RNA extraction. cDNA was synthesized using the HiScript[®] II first Strand cDNA Synthesis Kit (Vazyme, Nanjing, China), which incorporates a gDNA removal step to preclude genomic DNA contamination. Quantitative real-time PCR (qRT-PCR) was then performed on a

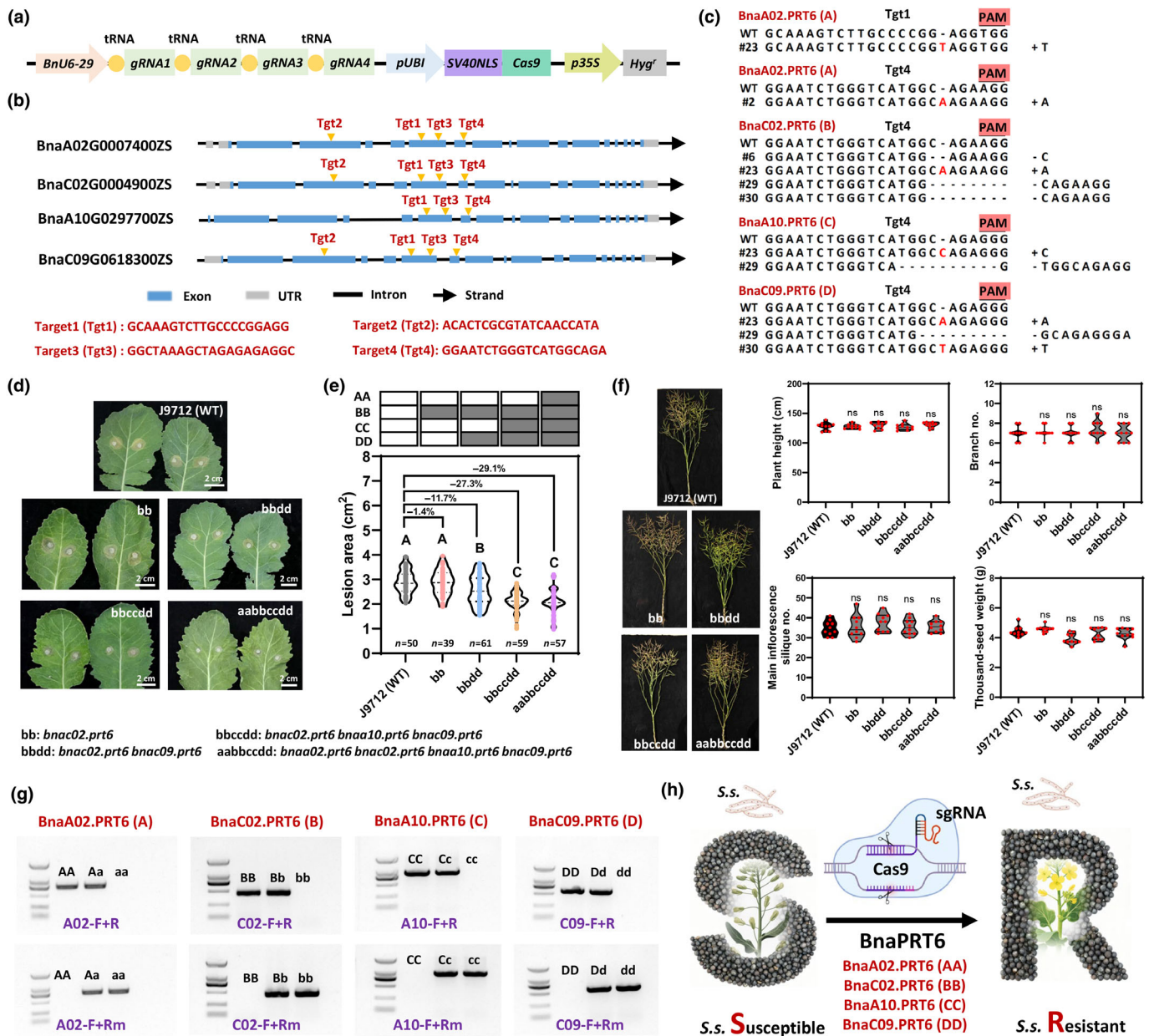


Fig. 2 Genetic knockout of *BnaPRT6* significantly enhances host resistance against *Sclerotinia sclerotiorum* (S.s.) in *Brassica napus*. (a) Schematic model of CRISPR/Cas9-mediated genome editing strategy targeting *BnaPRT6* genes. (b) Identification and target (Tgt) sequences of four *BnaPRT6* genes for CRISPR/Cas9 editing. (c) Sequences at the sgRNA target sites of *BnaPRT6* genes in the T₀ generation according to PCR and Sanger sequencing. Dashes represent deletions; red letters represent insertions. The protospacer-adjacent motif (PAM) is underlined. (d) Representative images of S.s. inoculation assays using various *bnprt6* mutant lines. (e) S.s. inoculation test in mock-treated and S.s.-infected areas. Data are presented as mean $\pm$ SE ($n = 39-61$, ANOVA with Tukey's *post hoc* test, $P < 0.01$) from three independent experiments. Dotted lines indicate the quartiles, while the dashed lines indicate the median. (f) Key agronomic traits between mutants and wild-type plants (J9712), including plant height, branch number, main inflorescence silique number and thousand-seed weight. ns, not significant ($n = 10$, $P > 0.05$, Student *t*-test). Quartiles and the median are shown by dotted and dashed lines, respectively. (g) Allele-specific markers were developed to discriminate the wild-type, heterozygous, and homozygous mutants for BnaA02.PRT6 (AA, Aa, and aa), BnaC02.PRT6 (BB, Bb, and bb), BnaA10.PRT6 (CC, Cc, and cc) and BnaC09.PRT6 (DD, Dd, and dd). (h) Schematic diagram of generating S.s.-resistant *B. napus* via CRISPR/Cas9-mediated knockout of *BnaPRT6*. The images were generated with online software (<https://www.doubao.com/>).

StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems Inc.), in strict accordance with the manufacturer's protocols.

The genes *BnaActin7* (BnaC02G0037200ZS) and *BnaUBC10* (BnaA10G0098100ZS) were employed as internal reference controls for normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2002).

RNA-Seq analysis

RNA-Seq analysis was conducted as previously described with minor modifications (Lin *et al.*, 2024). Briefly, leaf samples from wild-type rapeseed plants (J9712) were either collected after 3 h of complete submergence in darkness, or after 12 h of inoculation with *S. sclerotiorum*, with three independent biological replicates for each condition. High-quality mRNA was extracted using the RNeasy Plant Mini Kit (Qiagen). RNA libraries were prepared and sequenced on an Illumina NovaSeq 6000 sequencer by Majorbio Company (Shanghai, China), generating paired-end reads of 2×150 bp. Raw data in FASTQ format were initially assessed for quality using FASTQC (v.0.11.9). The reads were then aligned to the reference genome using HISAT2 (v.2.2.0) with default parameters, utilizing the *B. napus* (ZS11.v.0) genome as the reference. Differentially expressed genes were identified with a threshold of $|\log_2(\text{fold change})| \geq 1$ and a false discovery rate (FDR) < 0.05 . Significance of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes terms was determined with FDR ≤ 0.05 . The hypoxia-responsive genes examined in this study were selected based on well-characterized transcriptional profiles previously established under hypoxic conditions (Mustroph *et al.*, 2009). We identified 43 genes in *B. napus* with predicted functions identical to the 49 core hypoxia genes previously reported in Arabidopsis (Table S4).

Assessment of *S. sclerotiorum* susceptibility

The *S. sclerotiorum* isolate SS-1 was cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 23°C in the dark. Inoculum was prepared by punching a mycelial agar plug with a diameter of 7 mm from the actively growing margin of a 2-d-old *S. sclerotiorum* culture. The leaves were collected for inoculation with *S. sclerotiorum* from the field. Mock-inoculated leaves were treated with 7 mm in diameter agar plugs as a control. The leaves of the *B. napus* plants were inoculated with *S. sclerotiorum* for 40 h. The susceptibility of Arabidopsis leaves to *S. sclerotiorum* was assessed using a slightly modified inoculation method. Briefly, 2-mm-diameter agar plugs collected from the actively growing margins of *S. sclerotiorum* cultures were placed onto the leaves and maintained for 20 h. The detached leaf inoculation method is a well-established, high-throughput approach commonly used for phenotypic evaluation of *S. sclerotiorum* response in rapeseed and other crops (Dong *et al.*, 2008; Lin *et al.*, 2024; Malhotra *et al.*, 2026).

The lesion area caused by *S. sclerotiorum* infection was determined at different time intervals with IMAGEJ software (Lin *et al.*, 2024). The detached leaves from the 4-wk-old Arabidopsis were used for *S. sclerotiorum* inoculation using a similar procedure.

Planar O₂ sensor

The Planar O₂ sensor system operates on the established principle of fluorescence quenching (Holst & Grunwald, 2001; Meier *et al.*, 2011; Schreml *et al.*, 2011; Lenzewski *et al.*, 2018)

and utilizes a wavelength-ratiometric calibration method. The sensor incorporates an indicator dye and a reference dye, both immobilized in a polymer matrix to form a sensitive layer. The interaction between oxygen molecules and the indicator dye leads to quenching of its red fluorescence, and as a result the luminescence intensity of the indicator dye decreases with increasing oxygen content in the sample, whereas the reference dye remains unaffected by oxygen and emits a stable fluorescence signal.

This system comprised an O₂-sensitive foil (SF-RPSU4; PreSens GmbH, Regensburg, Germany) and a detection unit (VisiSens USB microscope; PreSens GmbH). Following a 28-h pre-inoculation with *S. sclerotiorum*, the fungal plug was removed from the leaf samples. The inoculation site was then immediately covered with an O₂-sensitive foil, which was secured in place using two plastic clamps. Leaf images were captured without delay using a dedicated detection unit. Non-inoculated areas on the same leaf served as mock controls. Technical specifications of the system are detailed in Tschiersch *et al.* (2012). All camera parameters were controlled via the VisiSens software (PreSens GmbH), following pre-calibration of the color channel sensitivity. Images were acquired at a resolution of 1280×1024 pixels (1.3 megapixels) in 24-bit color and saved in PNG format. The working distance was *c.* 2 cm, and the field of view measured $15 \text{ mm} \times 12 \text{ mm}$, yielding an effective pixel resolution of *c.* 12 μm . The actual spatial resolution was *c.* 20 μm , which was determined by the sensor characteristics and lateral O₂ diffusion within the sensitive layer. The software facilitated automated image acquisition and generated O₂ maps based on the raw sensor signals, using a two-point calibration procedure as described in the following section. The excitation light source and optical filters (excitation and emission) were selected to match the specific fluorescence response profile of the O₂-sensitive foil. Images were recorded at 10-s intervals over a total duration of 500 s.

Radial O₂ profiles

Leaves were excised from *B. napus* plants at the reproductive stage and inoculated with *S. sclerotiorum* by placing an agar plug containing fungal mycelium on the adaxial surface of the leaf. Inoculated leaves were kept moist and in darkness for *ca* 30 h before measurement. The agar plug was removed immediately before O₂ measurements to allow free diffusive gas exchange on both sides of the leaf. The 'mock' and 'infected' samples were taken from the same leaf. For each of the three leaves (S1, S2, and S3), O₂ levels were measured at three independent points within the infected region and three independent points within a mock-treated region on the same leaf. This was done to enable a direct paired comparison within each leaf. The two time points presented ($t = 0$ and $t = 1$) correspond to measurements taken immediately after treatment and 1-h post-treatment, respectively. An O₂ microsensor (OX-20; Unisense A/S, Aarhus, Denmark) was inserted *c.* 150 μm into the center of the lamina from the abaxial side, and O₂ levels were recorded using SensorTrace Suite v.3.4.800 (Unisense A/S).

Near-infrared spectroscopy analysis

The comparison of oil and protein content, fatty acid composition in mature seeds of J9712 (WT) and *BnaPRT6* knockout mutant lines was analyzed with near-infrared spectroscopy (NIRS) (NIRS DS2500F, Foss, Denmark).

Statistical analysis

Statistical analyses were performed by using MINITAB statistical software (v.17.0). Comparison of means was carried out for statistical significance using either ANOVA with Tukey's *post hoc* test or a two-sample Student's *t*-test. Before statistical analysis, data were verified for constant variance and normal distribution. Significance levels were set at *, $P < 0.05$, or **, $P < 0.01$.

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Competing interests

None declared.

Author contributions

CL, MJH and OP conceived and designed the project through collaborative discussions held via Zoom. YXL and ZZ performed most of the experiments. ZZ, XXM and QFS performed genetic transformation. DL, OP, XWS and JHZ performed the SSR resistance assays. CL, YXL and XWS analyzed the data. CL and OP wrote the manuscript with comments from all authors. HHL, FH, MJH and YPW revised the manuscript and gave suggestions. All authors read and approved the final manuscript.

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Data availability

The RNA-Seq data generated in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive database under accessions PRJNA1200603 and PRJNA1354449. Source data are provided with this paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Experimental setup for leaf inoculation with *Sclerotinia sclerotiorum* in *Brassica napus*.

Fig. S2 Experimental setup for O₂ level measurement with planar O₂ sensor and Microsensor in *Brassica napus*.

Fig. S3 Phylogenetic analysis and expression profiles of BnaPRT6 homeologs in *Brassica napus*.

Fig. S4 Identification of mutations by PCR followed by Sanger sequencing.

Fig. S5 The qRT-PCR analysis of seven selected hypoxia-related genes revealed differential expression between the *bnaprt6* quadruple mutant (aabbccdd) and the J9712 (WT) plants.

Fig. S6 The design of allele-specific molecular markers in *Brassica napus* mutants.

Table S1 Comparison of oil and protein content, fatty acid composition between different mutants and the wild-type cultivar J9712 in *Brassica napus*.

Table S2 Information on *PRT6* orthologs in plant species of the Brassicaceae family.

Table S3 Primers used in this study.

Table S4 The information of hypoxia-induced marker genes.

Video S1 Relative O₂ levels in mock-treated and *Sclerotinia sclerotiorum* infected areas with planar O₂ sensor.

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Key words: *Arabidopsis*, *Brassica napus*, genome editing, N-degron pathway, PROTEOLYSIS6, *Sclerotinia sclerotiorum*.

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