

Plant defensin gene PfPDF2 from *Perilla frutescens* confers high-level resistance to *Alternaria brassicae* and *Botrytis cinerea* in *Brassica napus*

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Abstract

Background

Plant defensins (PDF) are a group of small cysteine-rich peptides with broad-spectrum antimicrobial activity that play important roles in the response of plant to abiotic stresses.

Results

In this study, *Perilla frutescens* PDF (*PfPDF*) genes were identified and functionally characterized. A total of 6 *PfPDF* genes were successfully cloned. Characterization analysis revealed that all *PfPDF* genes consisted of 2 exons and 1 intron and encoded 73 ~ 88 amino acids, each of which contained 8 cysteine residues. Phylogenetic analyses indicated that all *PfPDF*s were members of the PDF type I family. The expression of *PfPDF* genes showed wide tissue specificity. *PfPDF1*, *PfPDF2* and *PfPDF4* were expressed in stems, leaves, flowers and seeds, and *PfPDF3* was expressed in leaves and seeds, while *PfPDF5* and *PfPDF6* were only expressed in seeds. Subsequently, *PfPDF2*, which was highly expressed in seeds, was transformed into *Brassica napus* to obtain transgenic plants. The total yield of transgenic *B. napus* was comparable to that of wild-type. Moreover, an *in vitro* antifungal bioactivity test on leaves revealed that the resistance to *Alternaria brassicae* and *Botrytis cinerea* was enhanced significantly in *PfPDF2*-overexpressing *B. napus* compared to that of the wild type. Analysis of differentially expressed genes in wild-type and transgenic *B. napus* showed that photosynthesis, fatty acid biosynthesis and metabolism pathways were significantly activated.

Conclusions

Overall, this study identified and characterized *PDF* genes from *P. frutescens* for the first time and clarified that *PfPDF2* could be applied to produce crops with higher resistance to pathogens, providing unique insights and new genetic resources for molecular breeding.

Background

In general, plants produce antimicrobial peptides (AMPs) to defend against potential pathogens in a wide range of environments, and they are key signaling molecules for plant immune responses. AMPs are mainly categorized into cyclotides, defensins, hevein-like proteins, lipid transfer proteins, snakins and thionins [1].

Plant defensins (PDF, known as Def) are a group of small peptides rich in cysteine residues and widely found in the roots, stems, leaves, flowers and seeds of cruciferous, legume and nightshade species [2, 3]. PDF has well-characterized antifungal activity, is nontoxic to plant and mammalian cells [4, 5], and can be considered an antibiotic compound with promising biotechnological applications. The primary structure of PDF proteins has less identity, but has a highly-conserved tertiary structure (1 α -helix and 3 β -sheets), that forms a distinctive cysteine-stabilized $\alpha\beta$ (CS $\alpha\beta$) motif. The CS $\alpha\beta$ motif was applicable in

plants, insects and fungi, and has strong stability [6, 7]. Stereospecific multiple sequence alignment revealed that a specific γ -core motif in the PDF, consisting of $\text{GXCX}_3\text{-}_9\text{C}$ sequence [8], and it has been proved to be necessary for antifungal and antibacterial activities.

PDF genes have been identified and successfully introduced into cash crops that enhanced the resistance of genetically modified crops to various pathogens. Over-expression of tobacco (*Nicotiana megalosiphon*) *NmDef02* was able to enhance the resistance of transgenic potato plants to late blight pathogen (*Phytophthora infestans*) [4]. Similarly, *NmDef02* could also improve the resistance of transgenic soybean to Asian soybean rust pathogen (*Phakopsora pachyrhizi*) and anthracnose pathogen (*Colletotrichum truncatum*) [9]. Expression of *AtPDF* contributes to preinvasive resistance to *Colletotrichum tropicale* in *Arabidopsis* [10]. Over-expression of poplar (*Poplar trichocarpa*) *PtDef* increases resistance to *Septotia populiperda* in poplar (*Populus* $\times$ *euramericana*), and speculated that *PtDef* plays an important role in activating the downstream transcription factor *MYC2* [3]. Meanwhile, *PDF* genes have been well characterized in many species, such as rice [11], tomato (*Solanum lycopersicon*) [12] and maize (*Zea mays*) [13]. Recently, it has been found that PDF could also be involved in the abiotic responses. In *Arabidopsis thaliana*, *AtPDF2.1* could mediate ammonium metabolism by regulating the activity of glutamine synthetase [14]. *AtPDF2.6* reduces the content of Cadmium (Cd) ion in the cytoplasm by chelation, thereby conferring Cd tolerance [15]. In rice (*Oryza sativa*), *OsCAL2*, a PDF protein localized in the cell wall, is regulating the Cd accumulation in transgenic *Arabidopsis* and rice shoots [2]. It is evident that *PDF* are high-quality candidate genes of the production for resistance to pathogen infection and remediation of heavy metal-contaminated soils.

Allotetraploid rapeseed (*Brassica napus*, $2n = 38$) is a widely cultivated oilseed crop worldwide, crucial in the food and feed industries, such as oil, biodiesel, bioethanol and animal feed. *Alternaria brassicae* is a serious pathogen causing black spot disease of *B. napus* [16]. An airborne plant pathogenic fungi, *Botrytis cinerea*, can be parasitic on organs such as flowers, leaves and pods of *B. napus*, and result in the development of gray mold [17]. These diseases significantly reduced the yield of *B. napus*, cause greatly economic losses, and should be controlled. It is well known that chemical control is not only harmful to the environment, but the use of same drugs may lead to the development of resistance in pathogens, and most importantly, there is a lack of appropriate predictive methods to ensure the regulated application of fungicides. Therefore, genetic engineering to breed new varieties of *B. napus* with higher resistance to pathogens is particularly important.

The dicotyledonous annual plant *Perilla frutescens* is widely planted in China as an oilseed crop [18]. There are few reports about *P. frutescens* being affected by *A. brassicae* and *B. cinerea* [19]. Previous research has shown that co-expression of *Rs-AMP1* (*PDF* gene) and *chit42* (chitinase gene) could enhance resistance to *Sclerotinia sclerotiorum* in *B. napus* [20]. The function of *PDF* genes has not yet been studied separately in *B. napus*. In the present study, 6 *PfPDF* genes were isolated and characterized from *P. frutescens*, and successful expression of *PfPDF2* gene in *B. napus*. Our results showed that *PfPDF2* enhances resistance to *A. brassicae* and *B. cinerea* pathogens without affecting yield traits in

oilseed rape, and provides a reference for breeding fungus resistant *B. napus* varieties through genetic engineering.

Materials and methods

1.1 Plant material, bacterial strains, and pathogen cultures

Annual *P. frutescens* seeds were sown in an outdoor experimental plot of the College of Life Sciences, Chongqing Normal University, Chongqing, China (29°36'50" N, 106°18'2" E) in April, and different tissues at flowering stage (roots, stems, leaves, and flowers) and mature seeds were collected in July, snap-frozen in liquid nitrogen and stored in -80°C refrigerator until use. *B. napus* (Zhongshuang No. 11, WT) seeds were sterilized with 75% ethanol for 30–60 s, then 0.1% mercuric chloride for 10 min, and finally washed 4–6 times with sterile double distilled water (ddH₂O). The sterilized *B. napus* seeds were germinated on 1/2 Murashige and Skoog (MS) medium (Coolaber, Beijing) and cultured at 25°C for 4–6 d in the dark condition to obtain asepsis seedling.

The competent *Escherichia coli* DH5α strain and *Agrobacterium tumefaciens* GV3101 strain were purchased from WEIDI (China) and used. To obtain vigorously growing pathogens, the pathogenic fungi (*A. brassicae* and *B. cinerea*) were inoculated in potato dextrose agar (PDA) medium, respectively, and then incubated at 26/26 °C (Light/Dark), 0 h/24 h (Light/Dark) conditions for 7 d.

1.2 Cloning and sequence characterization of PfPDF genes

Cloning primers were designed based on the sequences annotated as *PfPDF* genes in transcriptome database of *P. frutescens* [21], and then *PfPDF* sequences were cloned by PCR amplification using *P. frutescens* seed genomic DNA (gDNA) and cDNA as templates, respectively (Table S1). Each reaction comprised 1.5 µl template (10 ng), 12 µl Premix Taq (TaKaRa, Japan), 1.5 µl of the primers pairs (10 µM), and 10 µl ddH₂O. PCR was performed as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 60 s, and 72 °C for 10 min. PCR products were detected by 1% (w/v) agarose gel electrophoresis and collected using the SanPrep Column DNA Gel Extraction Kit (Sangon, China). The recombinant intermediate vectors pMD19T-*PfPDF* were constructed using the pMD19-T Vector Cloning Kit (TaKaRa, Japan) according to the manufacturer's instructions. Primer synthesis and sequencing were performed by ThermoFisher (China) and Sangon (China), respectively.

The intron-exon structures of the *PfPDF* genes were visualized by the GSDS 2.0 server (<http://gsds.gao-lab.org/>). The ProtoParam (<https://web.expasy.org/protparam/>) was used to analyze the basic characteristics of PfPDF, including the number of amino acids (NA), molecular weight (MW), theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY) and instability index (II). The amino acids translation of *PfPDF* genes was performed using Vector NTI software. DiANNA server (<http://clavius.bc.edu/~clotelab/DiANNA/>) was used to predict the disulfide bond topology [22]. Analysis of transmembrane helices and signal peptides was performed using the TMHMM-2.0 and SignalP-6.0

resources in the DTU.dk service (<https://www.dtu.dk/>), respectively. The secondary structure and 3D-model of PfPDF proteins were executed by Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2>) [23].

1.3 Analysis of protein-protein interaction (PPI) network

PPI network provides an intuitive and rapid understanding of gene function, and is also important for regulatory relationships between family proteins. Based on this, we constructed a PPI network of PfPDF proteins using the information of *Arabidopsis* homologs in the String database (v11.5, <https://cn.string-db.org/>), with the following parameters: all PfPDF protein sequences and the organisms being *Arabidopsis*.

1.4 Phylogenetic analysis

The *Arabidopsis* AtPDF protein sequences were downloaded from TAIR database (<https://www.arabidopsis.org/>). Multiple sequence alignment of AtPDF and PfPDF proteins using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and visualized by Jalview. The evolutionary relationship among AtPDF and PfPDF was elucidated by constructing an unrooted phylogenetic tree by the Neighbor-Joining (N-J) method in MEGA X [24]. Various technical parameters (e.g., 1000 bootstrap replicates, Pairwise deletion and the JTT + G evolutionary model) were used to construct, and visualized with the iTOL website (<https://itol.embl.de>). Detailed accession numbers of AtPDF sequences were available in Table S2.

1.5 PfPDF expression pattern analysis

The real-time quantitative polymerase chain reaction (RT-qPCR) was performed for analysis the expression pattern of PfPDF genes. Total RNA was extracted from different tissues (roots, stems and leaves, and flowers at flowering stage) and mature seeds of *P. frutescens* using the RNAprep pure plant kit (Tiangen, China), and then reverse transcribed to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Beijing). Efficient RT-qPCR primers were designed based on the coding sequences (CDS). The reaction system was 10 μ L SYBR Green super mix (Bio-Rad, USA), 1 μ L primer pairs, 4.0 μ L cDNA, and 5.0 μ L Nuclease-free H₂O. RT-qPCR reaction conditions were performed as follows: 95 °C for 60 s, followed by 40 cycles of 95 °C for 10 s 60 °C for 30 s. All RT-qPCR reactions were performed on a CFX96 Touch Real-Time PCR system (Bio-Rad, USA) and *Pf18S* as an internal reference gene. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression.

1.6 Construction of plant expression vector

The pCAMBIA1303 vector was double digested using *Nco*I and *Spe* , and purified *PfPDF2* fragment was ligated to the digested linearized pCAMBIA1303 vector using In-Fusion HD Cloning Kit (TaKaRa, Japan) in accordance with the manufacturer's instructions. The recombinant vector was transferred into DH5 α , and the positive strain was picked for sequencing. Finally, plasmid was extracted using TIANprep Rapid Mini Plasmid Kit (Tiangen, China) and transformed into GV3101 using the freeze-thaw method.

1.7 Production of transgenic plants

The cotyledons of *B. napus* were co-incubated with *A. tumefaciens* infestation solution for 5 min, and then cultured on 1/2 MS medium in dark conditions. After 2 d, the cotyledons were transferred to the callus induction medium (MS contain 6-BA 1 mg/L, 2,4-D 1 mg/L, Kan 50 mg/L, and Cef 500 mg/L) and cultured for 21–28 d. Subsequently, transferred into bud induction medium (MS contain 6-BA 3 mg/L, Cef 500 mg/L, ZT 2 mg/L, and Kan 20 mg/L). Transfer to stem induction medium (MS contain 6-BA 0.05 mg/L, Cef 250 mg/L, and Kan 20 mg/L) after it has grown young shoots. Finally, they were transferred to root induction medium (MS contain IBA 1 mg/L and Cef 50 mg/L) and cultured for 7 d. The well-grown regenerated seedlings were transplanted into plastic boxes with nutrient soil and coarse soil (1:2, v:v) after acclimatizing and grown at 25 °C with a photoperiod of 16/8 h (Light/Dark) until mature seeds were harvested.

The binary vector pCAMBIA1303, which carried a gene encoding hygromycin phosphotransferase as plant selection marker and β -glucosidase (GUS) as a reporter, and driven by the CaMV 35S promoter. The gDNA were extracted from different lines of T₁ generation transgenic *B. napus* and molecular identification using primers targeting the T-DNA region of pCAMBIA1303 (Table S1), gDNA of WT was used as a negative control. PCR was performed as follows: 94°C for 5min, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and 72°C for 10 min. The leaves of transgenic *B. napus* plants were stained and identified using the GUS stain Kit (Coolaber, China). Finally, total RNA was extracted from young leaves of transgenic and WT *B. napus*, and the expression level of *PfPDF2* was analyzed by RT-qPCR. The T₁ generation seeds were screened by 1/2 MS medium (20 mg/L hygromycin B), and transplanted to soil. The above procedure was repeated, the T₄ generation transgenic *B. napus* without trait segregation was considered as a homozygous line for further studies.

1.8 Resistance of *B. napus* expressing PfPDF2 to fungal pathogens

In vitro leaf antifungal bioactivity was measured on T₄ generation transgenic *B. napus* (A1, A2) and WT (50 days after sown). A 0.5 cm² wound was created on the adaxial surface of *B. napus* leaf through a scalpel, and a PDA block with actively growing pathogens (0.5 cm²) was placed on the proximal surface of the wound. Subsequently, co-cultured in an artificial incubator: 24 °C/20 °C (Light/Dark), 0 h/24 h (Light/Dark), 80% RH. Observed the disease symptoms and degree of damage after 7 DPI (days post inoculation). The farthest distance from the inoculation center to the lesion was recorded as lesion radius. The experiment was repeated four times.

1.9 RNA-Seq and KEGG analysis

Total RNA was extracted from leaves of 1-month-old WT and A2 plants, and cDNA libraries were prepared by the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, England) in accordance with the supplier's instructions, and subsequently sequenced by Illumina. Low quality bases (Qphred < = 20), adapters and lower quality sequences were removed from the raw sequence reads. The number of reads mapped to each gene was then calculated by featureCounts (1.5.0-p3) and the fragments per kilobase of transcript

per million mapped reads (FPKM) mapped to that gene was calculated. Finally, KEGG analysis of the differentially expressed genes (DEGs) were performed by clusterProfile [25].

1.10 Agronomic trait analysis

To determine whether the over-expression of *PfPDF2* function might affect agronomic traits, WT, A1 and A2 plants were grown in plastic boxes with the same nutrient soil under natural short-day (NSD, < 14-h light/day) conditions. Ten plants of each line were randomly selected before plant harvest to measure plant height and number of branches. The number of seeds per pod, 1000-grain weight, total yield and the number of pods per 20 cm were measured after maturity. The total protein content of A1, A2, and WT *B. napus* was determined by using a Protein Content (SP) Kit (Grace, China).

To determine whether the introduction of *PfPDF2* would have an effect on the production of *B. napus* seed oil, we also extracted the total oil from mature seeds to analyze oil content and fatty acid (FA) composition. Firstly, mature seeds were dried at 50°C for 48 h. The total oil of WT, A1 and A2 were extracted using the Soxhlet extraction method with petroleum ether (30–60°C) as solvent. The FA composition of the seed oil was analyzed by gas chromatograph (GC) according to Zhou et al [26].

1.11 Statistical analysis

The experimental results were statistically analyzed using Student's *t*-test in IBM SPSS Statistics. * and ** indicates significant difference at $P < 0.05$, $P < 0.01$, respectively. Graphs were drawn by using GraphPad Prism v8.0.

Results

2.1 Cloning and characterization of PfPDF genes

The complete open reading frames (ORF) of 6 *PfPDF* genes were obtained by using *P. frutescens* seed DNA as a template. Similarly, the full-length CDS were obtained by using *P. frutescens* seed cDNA as a template (Fig. 1A), and named *PfPDF1* to *PfPDF6*. The CDS of *PfPDF* genes ranged from 222 to 267 bp and encoded proteins were 73 to 88 amino acids. Gene structures analysis showed that all *PfPDF* genes consisted of 2 exons and 1 intron (Fig. 1B). *PfPDF1* and *PfPDF3* have larger intron sequences. Conserved domains analysis showed that PfPDF proteins are members of the gamma-thionin family and contain a Knot1 domain, which confirms the authenticity of the PfPDF sequences (Fig. S1).

The basic properties of the 6 PfPDF were analyzed by ProtoParam. All the 6 PfPDF are basic peptide, and Mw of PfPDF ranges from 7.82 to 9.29 kDa, and the *pI* ranges from 8.95 to 9.63. The GRAVY values indicated that PfPDF1 to PfPDF5 are hydrophobicity, while PfPDF6 is hydrophilicity. The II results showed that PfPDF2, PfPDF4, PfPDF5, and PfPDF6 were unstable proteins, while PfPDF1 and PfPDF3 were stable proteins (Table 1). Signal peptide prediction indicated that all PfPDF contain an N-terminal signal peptide, which plays an important role in guiding the transmembrane transfer of newly synthesized

proteins (Fig. S2). Topological analysis showed that all PfPDF members have one transmembrane domain (Fig. S3).

Table 1
Physicochemical properties of PfPDF proteins

Name	Na	Mw (Da)	pI	GRAVY	II
PfPDF1	74	8321.91	9.63	0.193	27.32
PfPDF2	88	9287.83	9.42	0.002	55.17
PfPDF3	73	7822.15	8.95	0.130	43.07
PfPDF4	76	8695.52	9.34	0.017	30.69
PfPDF5	75	8449.98	9.49	0.029	41.63
PfPDF6	76	8572.24	9.30	-0.020	57.51

2. Multiple sequence alignment and phylogenetic analysis

The amino acid sequence similarity analysis revealed that the identity between PfPDF and AtPDF was 19.44 to 61.11%. The identity of the PfPDF members ranged from 38.24 to 56.94%, while identity of AtPDF members ranged from 22.22 to 91.25% (Table S3), indicating that a low identity of amino acid sequences in different or homologous PDF proteins. Multiple sequence alignment of amino acids from PfPDF and AtPDF proteins showed that PfPDF have 8 conserved cysteines which form a conserved CSαβ-core motif and γ-core motif (Fig. 2). The predicted results of disulfide bond topology indicated that all PfPDF have typical linkage forms (1–8, 2–5, 3–6, and 4–7) and form a distinctive CSαβ motif consisting of 1 α-helix and 3 β-sheet (Fig. 2). In addition, 3 typical conserved amino acids, 2 Gly and 1 Ser, are also present. EGF_{XGG} (74th to 79th) and RGF_{RRR} (85th to 90th) elements are also conserved. 3D-models of PfPDF showed that all PfPDF proteins have similar structures (Fig. S4).

An unrooted phylogenetic tree was constructed based on 6 PfPDF and 11 AtPDF protein sequences to further explore the evolutionary relationship of PfPDF. The topological branches of phylogenetic tree grouped 17 PDF proteins to PDF1 and PDF2 family, and all PfPDF are members of PDF2 family (Fig. 3).

3. Analysis of PPI network in PfPDF proteins

In order to investigate the potential mechanisms of PfPDF genes against pathogens, a PPI network was constructed through the STRING database (Fig. 4). The PPI network consists of 15 protein nodes and 29 edges, and the average local clustering coefficient was 0.624, indicating that the PPI network have a high degree of credibility. The PfPDF proteins are widely involved in biological processes such as defense response (fungus and other organisms), killing of cells of other organisms, phyloquinone, and salicylic acid biosynthetic. PPI network showed that PfPDF1 to PfPDF5 potentially interacted with 3, 5, 8, 3, and 3 proteins, respectively, while PfPDF6 had no interaction protein. PfPDF2 interacts with SBT4.9, MIOX5,

LCR59 and LCR80. We speculated that this process plays an important role in resistance to pathogenic infestation in *P. frutescens*.

4. Tissue-specific analysis of PfPDF genes

To investigate the potential function of *PfPDF* genes in the development process of *P. frutescens*, the expression level of *PfPDF* genes in different tissues were detected by RT-qPCR. The results showed that *PfPDF1*, *PfPDF2* and *PfPDF4* genes were expressed in stems, leaves, flowers and seeds. *PfPDF3* gene was expressed in leaves and seeds, and *PfPDF5* and *PfPDF6* genes were only expressed in seeds (Fig. 5). The expression of *PfPDF* genes in roots was not detected, and we presumed that they do not function in the root or only function at a specific time. *PfPDF2* expressed in all tissues except roots and highly expressed in seeds, therefore, we conducted a follow-up in-depth study on *PfPDF2* gene.

5. Production of transgenic *B. napus*

The recombinant vector pCambia1303-*PfPDF2* was constructed and transformed into *A. tumefaciens* GV3101, and finally transformed into *B. napus* according to *Agrobacterium*-mediated transformation method. After hygromycin B screening, GUS staining, PCR identification and self-pollination process, a total of 3 transgenic *B. napus* lines were obtained without trait segregation, named A1, A2, and A3 (Fig. S5A, B). Gene expression analysis identified that the *PfPDF2* gene has expressed in transgenic *B. napus* (Fig. S5C).

6. Enhanced fungal resistance in transgenic *B. napus* against *A. brassicae* and *B. cinerea*

In order to determine whether the expression of *PfPDF2* conferred resistance against *A. brassicae* and *B. cinerea* pathogens, *in vitro* leaf antifungal bioactivity was performed. After 7 DPI of *A. brassicae*, the lesion radius on *in vitro* leaves of A1 and A2 lines was much less than that of WT, being 27.42% and 12.33% to that of WT, respectively (Fig. 6A-C, Table 2). Meanwhile, after 7 DPI of *B. cinerea*, the leaves of WT were fully infected and yellowed, and the average lesion radius was 7.53 cm (Fig. 6D-F, Table 2). Nevertheless, *in vitro* leaves of transgenic *B. nups* received almost no effect by *B. cinerea*. The lesion radius of A1 and A2 lines was only 50% and 11.69% of WT, recorded 2.18 and 0.98 cm, respectively. Much less damage was observed in transgenic *B. napus* by fungus, indicated that the introduction of *PfPDF2* in *B. napus* increased resistance to *A. brassicae* and *B. cinerea*.

Table 2
Analysis of lesion radius between WT and transgenic *B. napus* lines.

Type	<i>A. brassicae</i>	<i>B. cinerea</i>
WT	7.53 ± 1.07	7.95 ± 0.93
A1	3.70 ± 0.22**	2.18 ± 0.39**
A2	0.88 ± 0.51**	0.98 ± 0.68**

Each value represents the mean ± S.D. (n = 4) for the WT, A1 and A2.

7. KEGG analysis of DEGs

To understand the potential molecular mechanisms of *PfPDF2* in regulating plant responses to *A. brassicae* and *B. cinerea*, we compare leaves of one-month-old WT and A2 plants by using RNA-seq. Compared to WT, we identified 18802 differentially expressed genes (7578 up-regulated and 11224 down-regulated) in A2 (Fig. 7A). Then, these genes were subjected to KEGG analysis, which showed that they were mainly enriched in the photosynthesis, fatty acid biosynthesis and metabolism, glycolysis/gluconeogenesis, and pentose phosphate pathway pathways (Fig. 7B).

8. Yield analysis of transgenic *B. napus*

To determine whether the introduction of the *PfPDF* gene affects the agronomic traits of *B. napus*, we transplanted A1, A2, and WT into soil-filled plastic boxes under NSD conditions. There was no significant differences in plant height and flowering time between transgenic and WT *B. napus*. The 1000-grain weight, number of branches and pods increased significantly in A1 and A2 compared to that of WT (Table 3, Fig. 8A-D), while the pod length and number of filled grains in transgenic *B. napus* decreased significantly compared to that of WT. Nevertheless, there was no significant difference in total yield between transgenic and WT *B. napus*, probably due to the increase of 1000-grain weight and number of pods.

Table 3
Comparative analysis of agronomic characters between WT and transgenic *B. napus* lines.

Type	Plant height (cm)	No. of branch (/Plant)	Number of pods	Pod length (cm)	No. of fill grains	1000-grain weight (g)	Yield (g/plant)
WT	166.91 ± 2.25	9.92 ± 0.51	426.60 ± 8.46	9.25 ± 0.16	28.33 ± 1.23	2.83 ± 0.13	34.14 ± 3.02
A1	167.42 ± 1.56	14.62 ± 1.56**	477.40 ± 9.45**	4.20 ± 0.18**	24.00 ± 1.00*	3.13 ± 0.29**	35.91 ± 2.53
A2	166.90 ± 1.93	11.52 ± 1.33*	463.70 ± 9.50**	4.12 ± 0.24**	24.71 ± 1.33*	3.12 ± 0.26*	35.70 ± 3.10

Each value represents the means $\pm$ S.D. (n = 10) for the WT, A1 and A2 lines.

9. Expression of PfPDF2 altered oil and protein content, and FA accumulation in *B. napus* seeds

Total oil was extracted from the mature seeds of A1, A2 and WT lines, and FA composition were analyzed by GC-MS. Seed oil content of WT line was 46.36%, while oil content of A1 and A2 lines decreased by 11.17% and 8.07%, recorded 41.18% and 42.62%, respectively (Fig. 9A). By contrast, the total protein content in seed of A1 and A2 was higher than that of WT, with an increase of 13.80 and 6.37 mg/g, respectively (Fig. 9B). Furthermore, over-expression of *PfPDF2* gene in *B. napus* significantly affected the FA composition. Compared to WT, content of stearic acid (C18:0) and oleic acid (C18:1) of transgenic *B. napus* increased by 23.39% and 5.03%, respectively. While the content of linoleic acid (C18:2), α -linolenic acid (C18:3), and cis-11-eicosenoic acid (C20:1) decreased by 10.25%, 37.97 and 39.51%, respectively (Fig. 9C).

Discussion

Crops are usually directly affected by insect pests and pathogens in natural environment, resulting in slowed growth, reduced yields, and substantial economic losses [27]. *PDF* genes play a variety of roles in response to stress, growth and development of plant, therefore, have been widely used in genetic engineering in the last decade. Currently, *PDF* genes have been identified and researched in several higher plant species, including *Arabidopsis* [10], tobacco [4], rice [11], tomato (*Solanum lycopersicon*) [12] and maize (*Zea mays*) [13], and overexpression of *PDF* showed highly resistance to pathogens. However, the *PfPDF* genes have not been identified and their biological functions are still unknown.

In this study, 6 *PfPDF* genes were obtained from *P. frutescens* seeds, which consists of cysteine-rich peptides with 73 ~ 88 amino acids. *PfPDF* consists of 2 exons and 1 intron, which is consistent with that of *B. napus* [28]. *PDF* proteins are classified into two categories based on the absence (class I) or presence (class II) of an acidic C-terminal propeptide (CTPP) of approximately 33 amino acids [29]. In this study, signal peptide and multiple sequence alignment analysis revealed that they all contain only one N-terminal signal peptide, and the 8th cysteine residue is followed by a termination codon, therefore, all *PfPDF* proteins belong to class I. The four disulfide bonds (1–8, 2–5, 3–6, and 4–7) in *PfPDF* proteins constitute the conserved CS α β motif (CX₁₀CX₅CX₃CX_{20–26}CX₃C), and were considered to be necessary for structure and function [6]. In addition, the γ -core motif (GXCX₆C) is also conserved in *PfPDF*, which confers antifungal activity and morphogenicity to plants [8, 30, 31]. The 6 *PfPDF* genes are members of *PDF2* family based on phylogenetic relationships.

Identification of potential proteins that interact with the *PfPDF* proteins is intuitive to understand its antimicrobial mechanism. The PPI network revealed that *PfPDF2* interacts with SBT4.9, MIOX5, LCR59, and LCR80. SBT4.9 is a member of the subtilisin-like protease family which is induced by pathogens. It is hypothesized that SBT4.9 undergoes hydrolysis events during pathogenesis of plant and its expression is up-regulated by salicylic acid [32]. Salicylic acid plays an important role as a key plant defense hormone

against pathogens [33]. The *miox1/2/4/5* quadruple mutants significantly reduced the susceptibility to beet cyst nematode (*Heterodera schachtii*) [34]. LCR59 and LCR80 are proteins that encode a pathogenesis-related protein, and are members of the PDF family. The PPI network of PfPDF with SBT4.9, MIOX5, LCR59, and LCR80 provides a unique insight into the mechanism of PfPDF defense against pathogens.

PDF genes are widely present in roots, stems, leaves, flowers and seeds of plants [2, 3, 35]. In this study, PfPDF genes were expressed in stems, leaves, flowers and seeds, suggesting that PfPDF genes were involved in different physiological processes of *P. frutescens*. Previous studies have shown that PDF proteins could inhibit growth of pathogens. *OsDEF7* and *OsDEF8* inhibited the growth of *Xanthomonas campestris* [11]. In model legume (*Medicago truncatula*), *MtDef5* binds to negatively charged, and may have the function of inhibiting DNA synthesis and transcription in the Gram-negative *Xanthomonas campestris* and its γ -core motifs have antibacterial and antifungal activity [36]. Compared with wild-type pepper (*Capsicum annuum*), over-expression of *J1-1* in *C. annuum* leads to restriction of fungal colonization by inhibiting the growth and spore production of *Colletotrichum gloeosporioides*, which prevented the spread of anthracnose symptoms in the immature *C. annuum* fruits [37]. Transgenic tobacco expressing the mustard (*Brassica juncea*) PDF gene (*BjD*) had enhanced resistance to *Phytophthora parasitica* and *Fusarium moniliforme*, meanwhile, expression *BjD* in peanut enhanced the resistance of isolated leaves to *P. personata* and *Cercospora arachidicola* [38]. Thus, it has been proven that PDF genes have a broad-spectrum antimicrobial activity to pathogens rather than limited to a single pathogen. In *Arabidopsis dwarf at early stages1 (dwe1)* mutant with enhanced expression of *PDF1.2a* and *PDF1.2b*, showed increased resistance to infection of *B. cinerea* [39]. We also found that expression of the PfPDF2 in *B. napus* significantly increased resistance to *A. brassicae* and *B. cinerea*.

B. napus often suffers from infection by pathogens *A. brassicae* and *B. cinerea* during growth and development, accompanied by a reduction in yield traits [17, 40]. Thus, we constructed transgenic *B. napus* expression of the PfPDF2 and analyzed the transcriptome effect of PfPDF2 gene on disease resistance by RNA-seq. In the present study, genes for enzymes associated with photosynthesis were the most abundant DEGs, an observation similar to previous findings [41, 42], suggesting that plants may defend against pathogens through reduced carbon capture. It should be noticed that the oil content of transgenic oilseed rape was reduced by 8.07 to 11.17% in our experiments, which could be attributed to the down-regulation of the fatty acid synthase gene. As far as we know, this is the first report that PfPDF gene function as resistance to fungal pathogens. The results of this study provide new insight into the functions of PfPDF and expand the variety and function of PDF against pathogenic fungi.

Conclusion

In this study, 6 PfPDF genes were isolated from *P. frutescens*, and their gene structure, phylogenetic relationship, PPI network and tissue-specific expression pattern were analyzed. PfPDF genes are all composed of a structure with 2 exons and 1 intron, and are members of the PDF1 family, which implies that PfPDF genes are relatively conserved during evolution process. Tissue-specific analysis showed that

the *PfPDF* genes play important roles in the growth and development of *P. frutescens*. The yield traits are not affected in *B. napus* expressing the *PfPDF2* gene. Furthermore, transgenic *B. napus* has enhanced resistance to *A. brassicae* and *B. cinerea*, suggesting that *PfPDF2* is an excellent candidate gene for crop quality improvement.

Abbreviations

PDF plant defensin

NA number of amino acids

MW molecular weight

pI theoretical isoelectric point

GRAVY grand average of hydropathicity

II instability index

MS Murashige and Skoog

Kan Kanamycin

Cef Cefotaxime

ZT Zeatin

IBA Indolebutyric acid

FA fatty acid

Declarations

Acknowledgments

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Authors' contributions

HX, TZ planned and designed the research, analyzed data, and organized the manuscript. XG, HB conducted experiments, data analysis, and wrote the manuscript. JY, HS, JL, BZ conducted data analysis. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

All plant materials were used following national and international standards and local laws and regulations. Using all plant materials does not pose any risk to other species in nature. No specific permission is required to collect all samples described in this study. The planting area of *Perilla frutescens* [*Perilla frutescens* (L.) Britt.] is our experimental plot, and the field collection does not involve endangered or protected species. All *Perilla frutescens* samples (roots, stems, leaves, flowers and seeds) were stored at the College of Life Sciences, Chongqing Normal University.

Consent for publication

Not applicable.

Data Availability statement

All data analyzed during this study are included in the supplementary information files, and genotypic data have been deposited in the Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) to NCBI under <https://submit.ncbi.nlm.nih.gov/subs/sra/SUB13167949/overview> or BioSample accession No. SAMN34358994.

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Figures

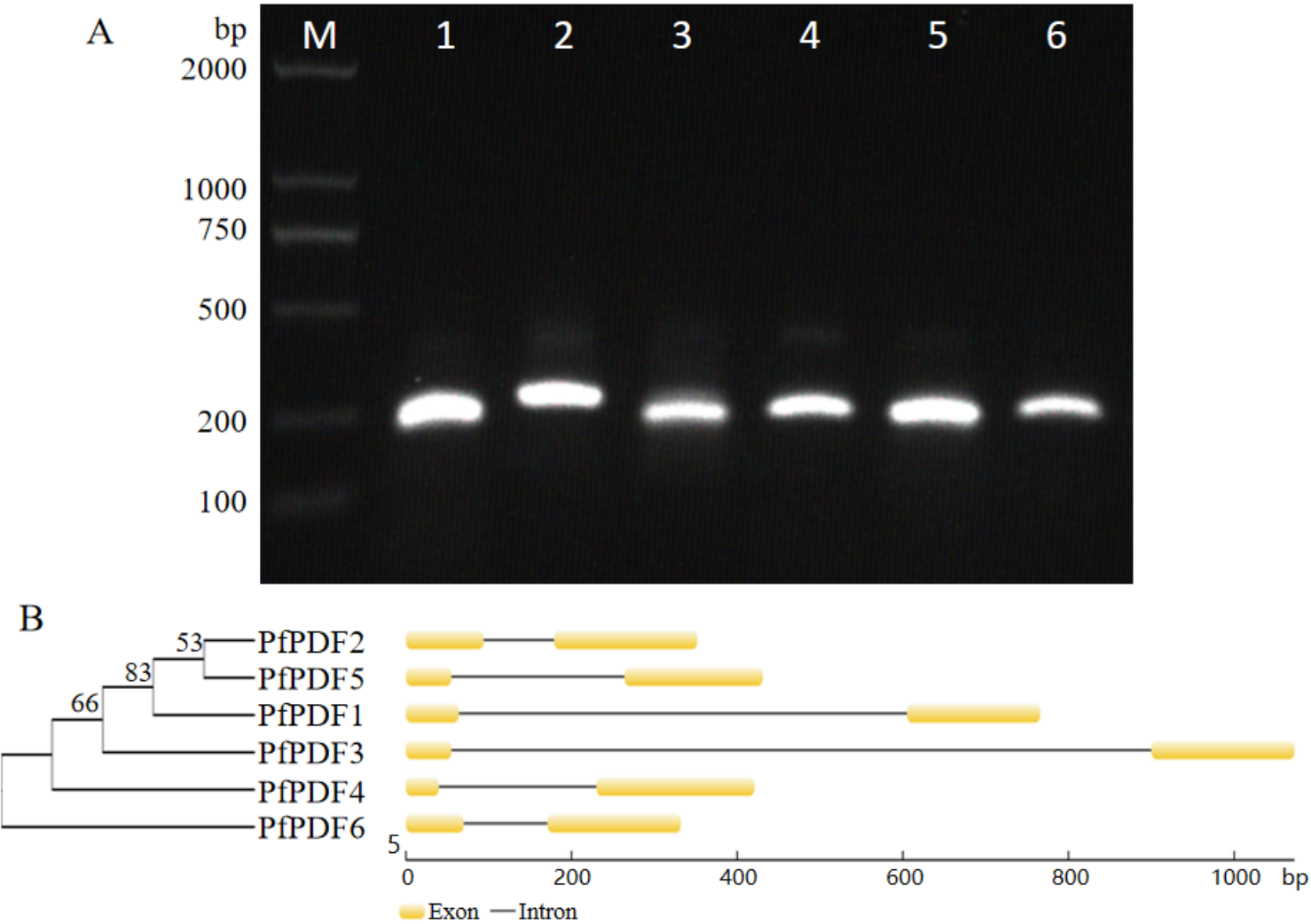


Figure 1

PCR products and structures of *PfPDF* gene. (A) 1-6: PCR amplification products of *PfPDF1* to *PfPDF6*, M: DNA Marker (100 to 2000 bp, from top to bottom were 2000, 1000, 750, 500, 250, and 2000 bp, sequentially). (B) Gene structures of *PfPDF* genes.

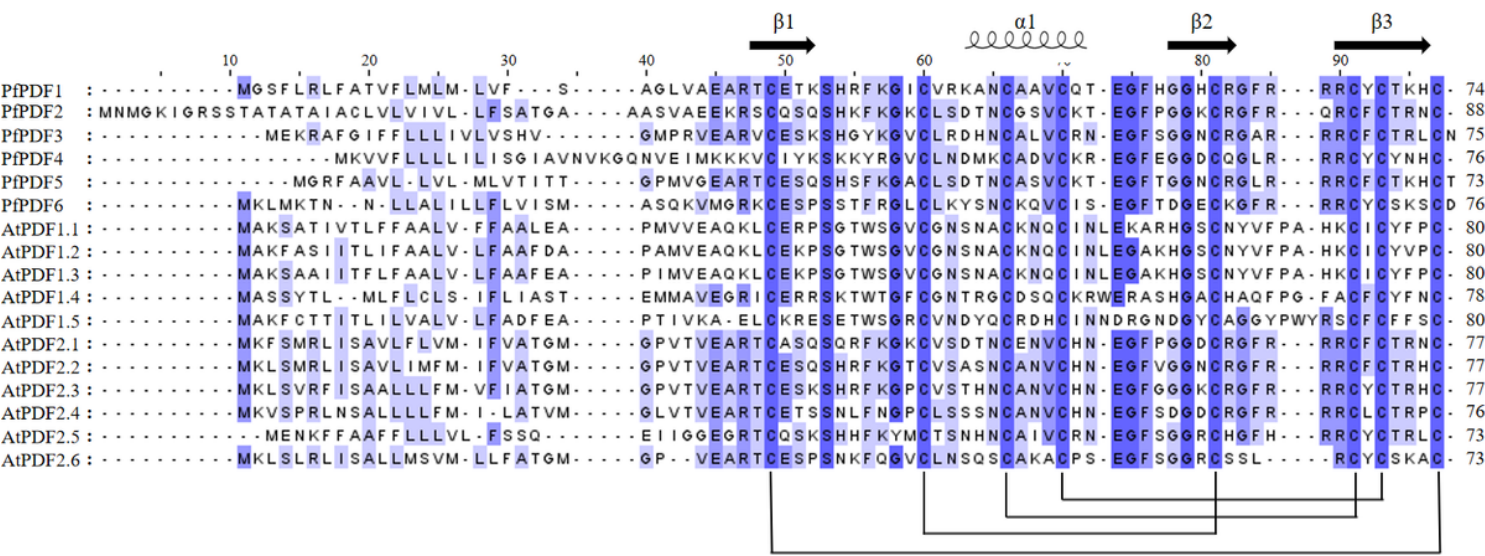


Figure 2

The amino acid sequence alignment of PDF proteins in *Arabidopsis* and *P. frutescens*. The alignment was performed using Clustal Omega. Jalview software was used for plotting. DiANNA was used for predicting disulfide bond topology. Phyre2 was used to determine the secondary structure, α -helix ($\alpha 1$), and β -sheet ($\beta 1$, $\beta 2$, $\beta 3$).

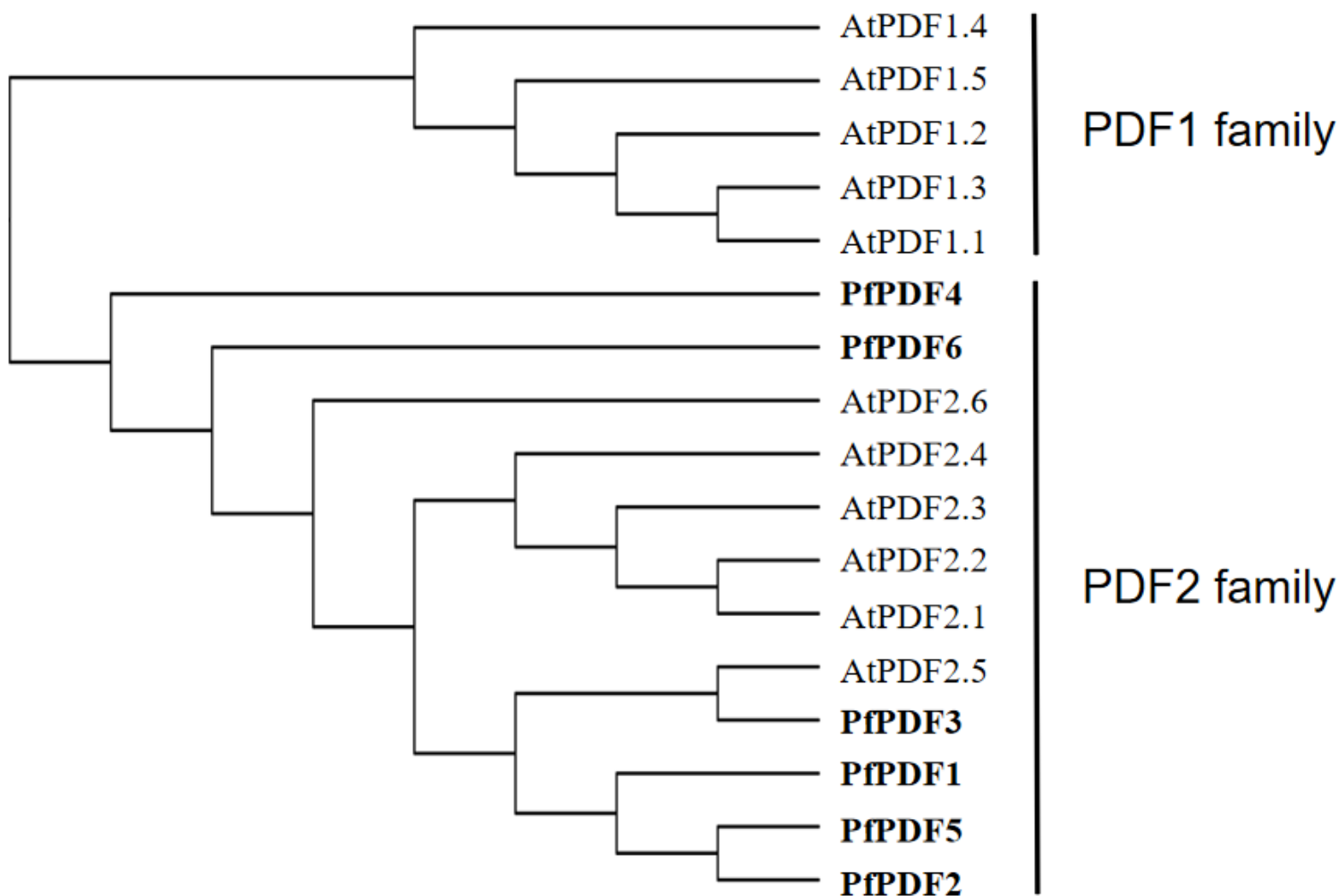


Figure 3

Phylogenetic tree showed the evolutionary relationship of PDF proteins in *Arabidopsis* and *P. frutescens*. The N-J method was used to build the phylogenetic tree using the MEGA X software. Detailed accession numbers of *Arabidopsis* AtPDF proteins were listed in Table S2.

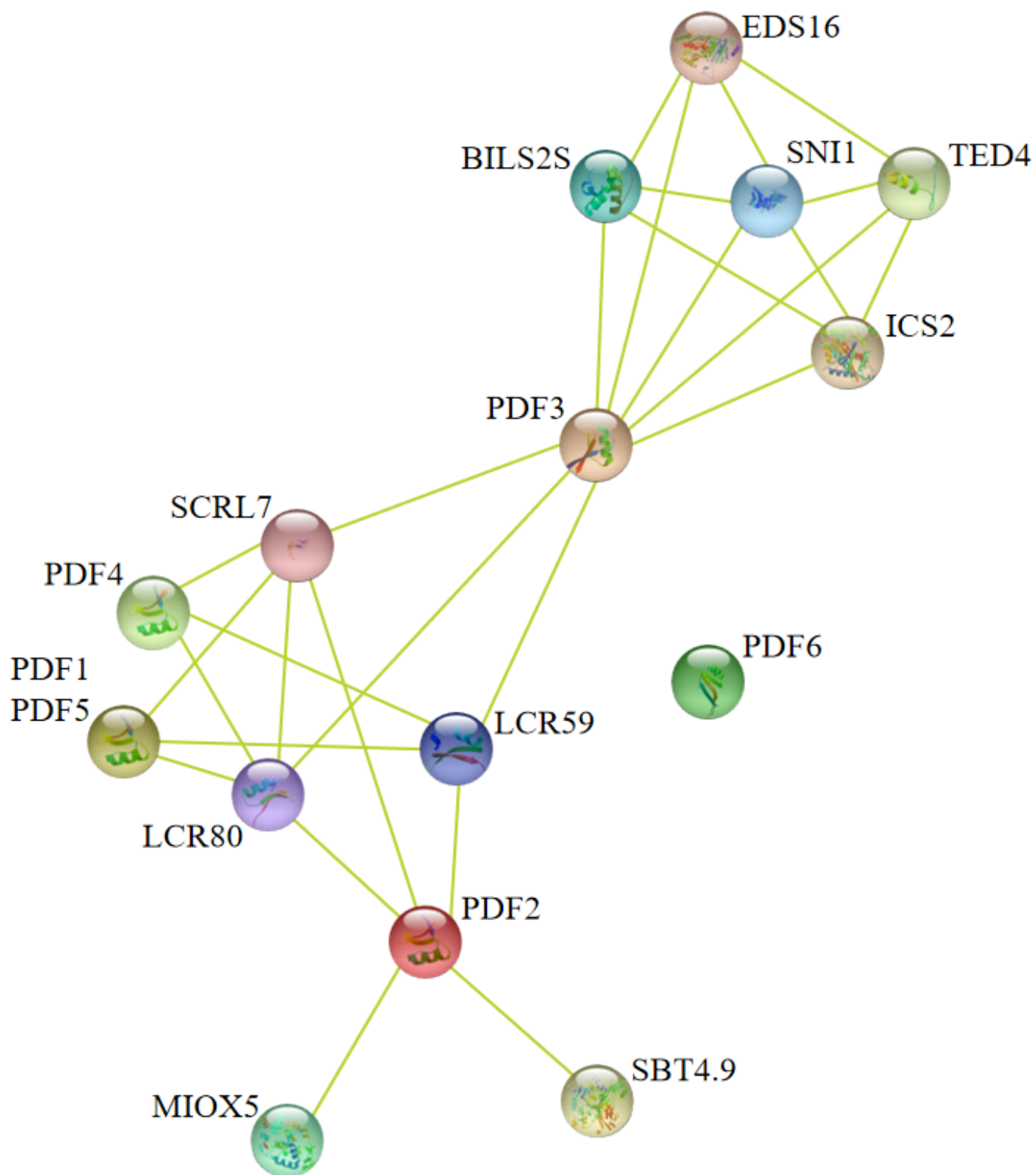


Figure 4

The PPI network of PdfPDF proteins was predicted by String. In this PPI diagram, one gene was represented by a circle, and the protein structure was displayed in the center of the circle. Abbreviations: EDS16, Enhanced Disease Susceptibility to Erysiphe Orontii 16; BILS 2S, Bifunctional Inhibitor/Lipid-transfer protein/Seed storage 2s Albumin Superfamily Protein; SNI1, Suppressor of NPR1-1; TED4, Tracheary Element Differentiation-related 4; ICS2, Isochorismate Synthase 2; PDF1 to PDF6, *P. frutescens*

plant defensin 1 to 6; SCRL7, Scr-Like 7; LCR59, Low-Molecular-Weight Cysteine-rich 59; LCR80, Low-Molecular-Weight Cysteine-rich 80; MIOX5, Myo-Inositol Oxygenase 5; SBT4.9, Subtilisin-Like Protease 4.9.

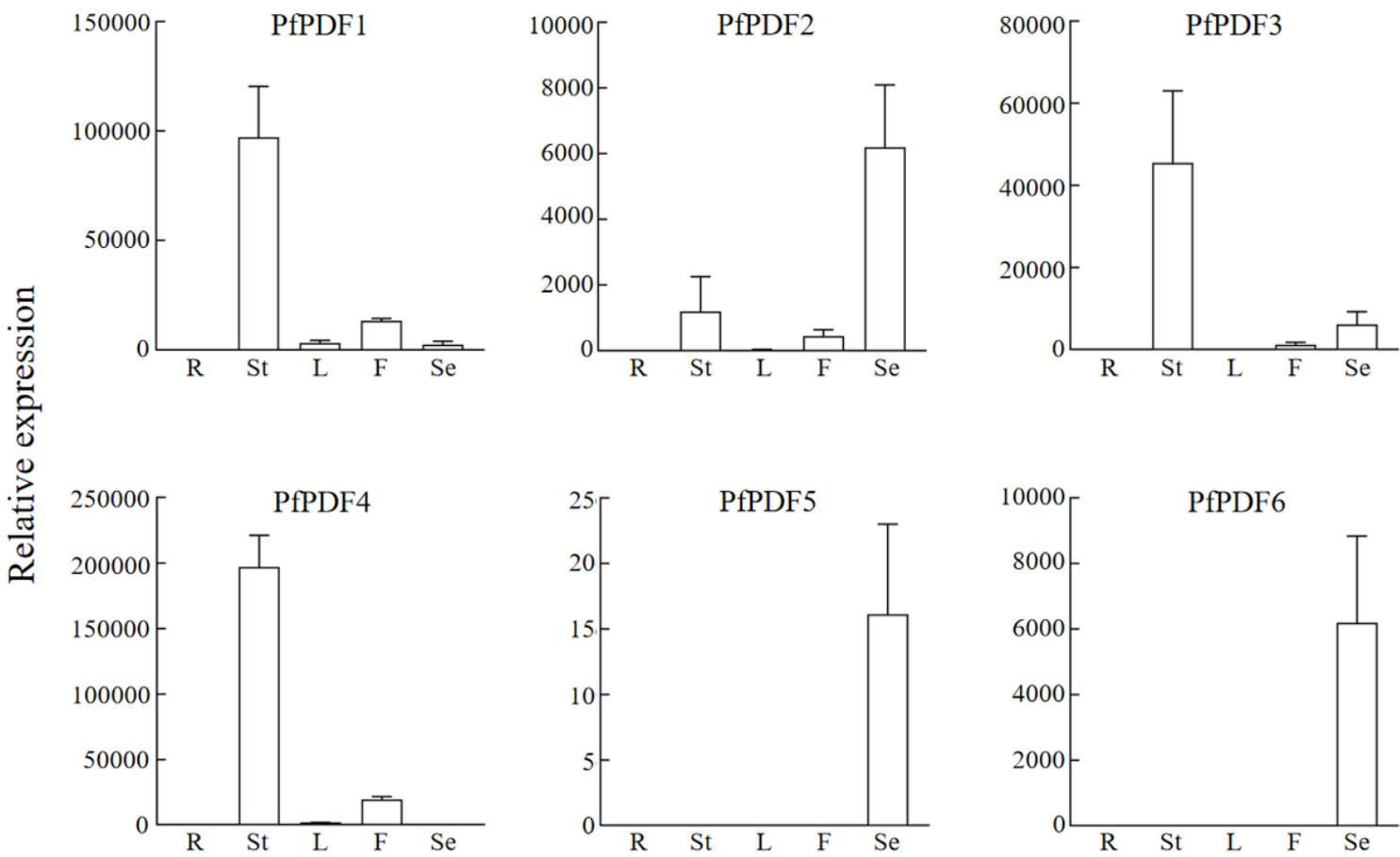


Figure 5

The expression patterns of *PfPDF* genes in different tissues of *P. frutescens*. R, roots; St, stems; L, leaves; F, flowers; Se, seeds. Data were represented as means ± SE of three experimental replicates and tree technological replicated.

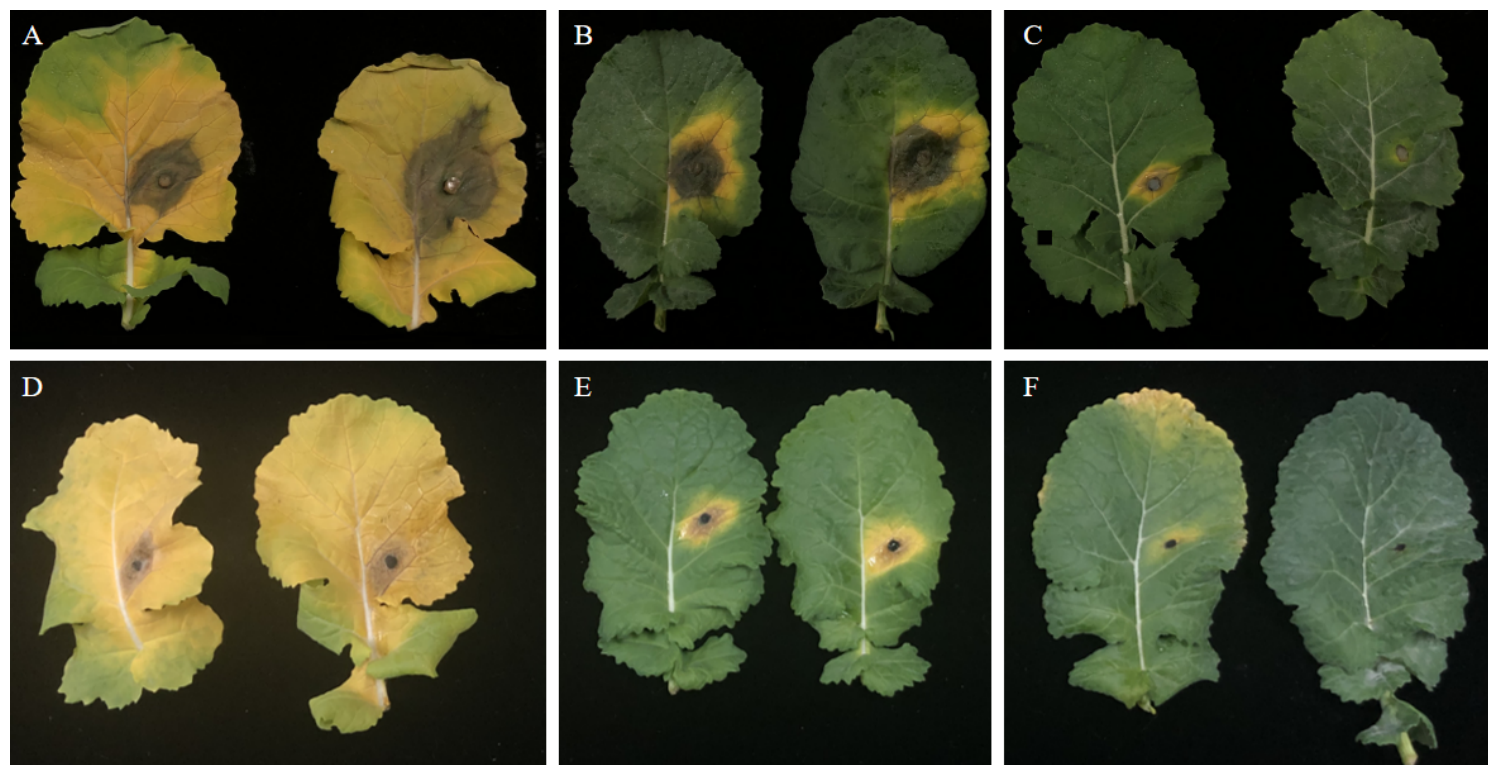


Figure 6

Analysis of the transgenic *B. napus* with the challenge from *A. brassicae* and *B. cinerea*. (A, B, C) Phenotypic observations of *In vitro* leaves of WT, A1, and A2 lines, respectively, 7DPI with *A. brassicae*. (D, E, F) Phenotypic observations of *In vitro* leaves of WT, A1, and A2 lines, respectively, 7 DPI with *B. cinerea*.

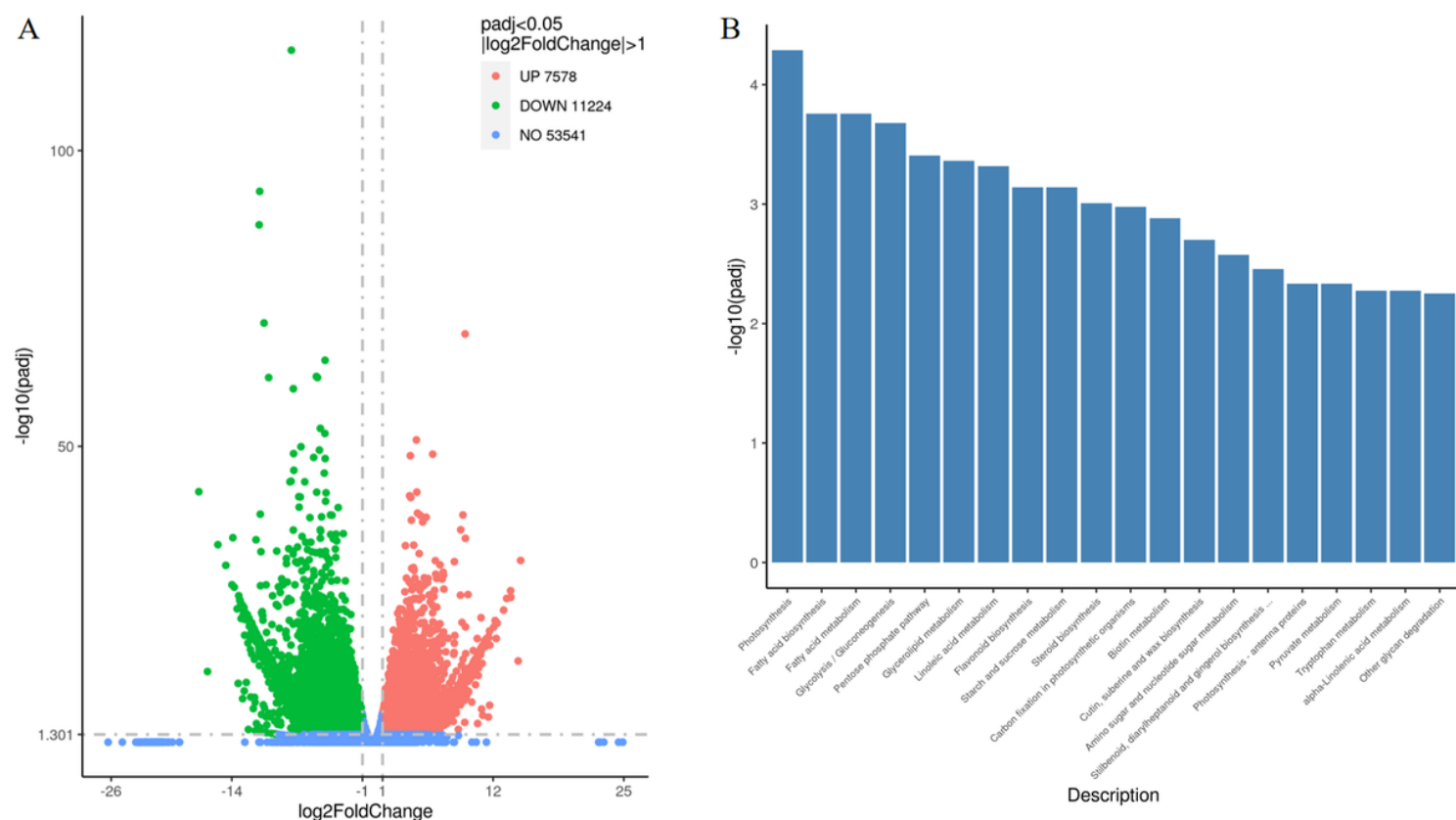


Figure 7

DEGs in the WT and A2 plant. Comparative analysis of A2 and WT by volcano plot (A) and KEGG (B)

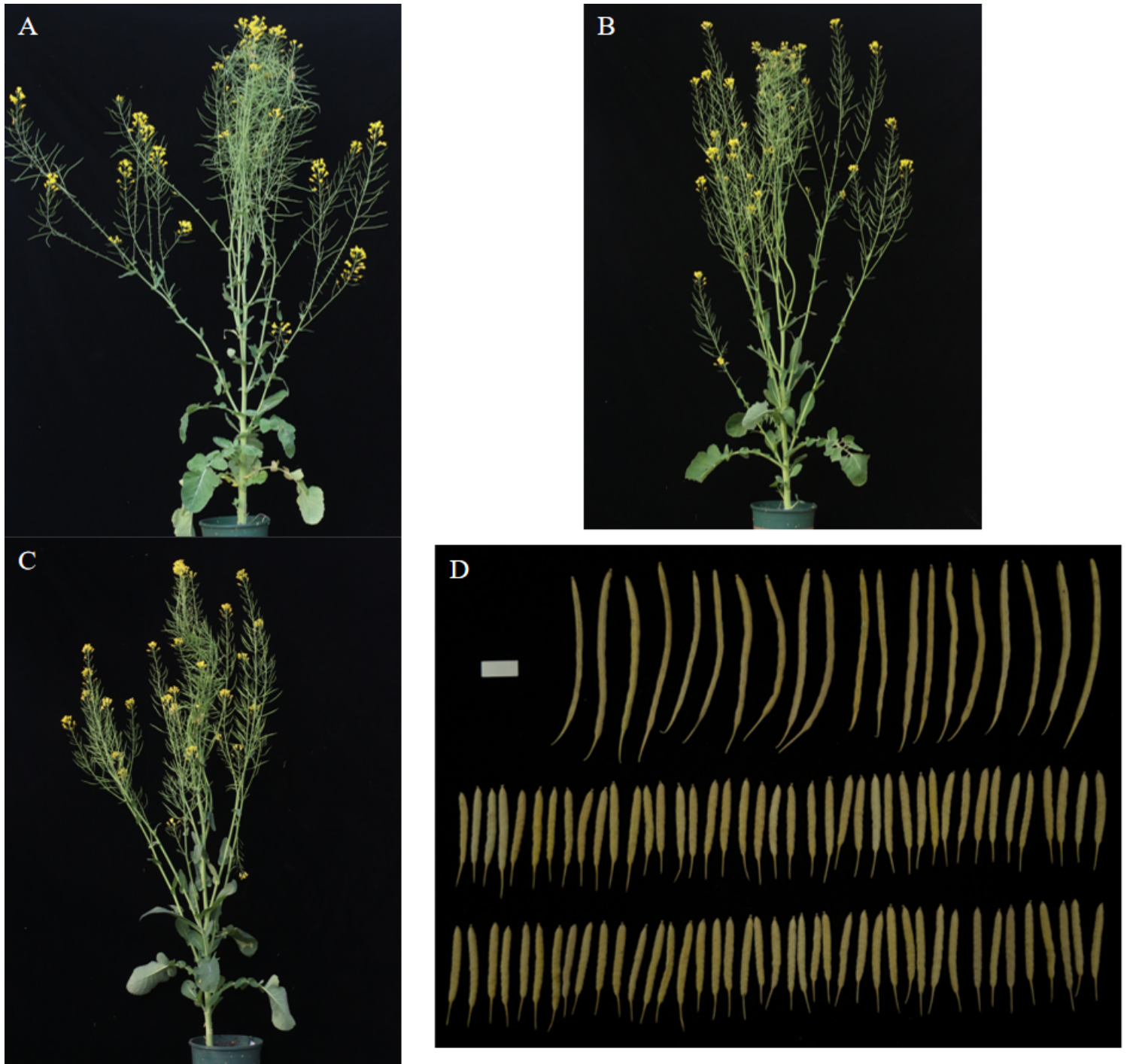


Figure 8

Phenotype observation of plant and seed between WT and transgenic *B. napus* lines. Phenotype observation of WT (A), A1 (B), and A2 (C) lines. (D) Phenotype observation of WT and transgenic *B. napus* in pods numbers (20 cm). From top to bottom were WT, A1, and A2, sequentially. The white bar represents 2 cm.

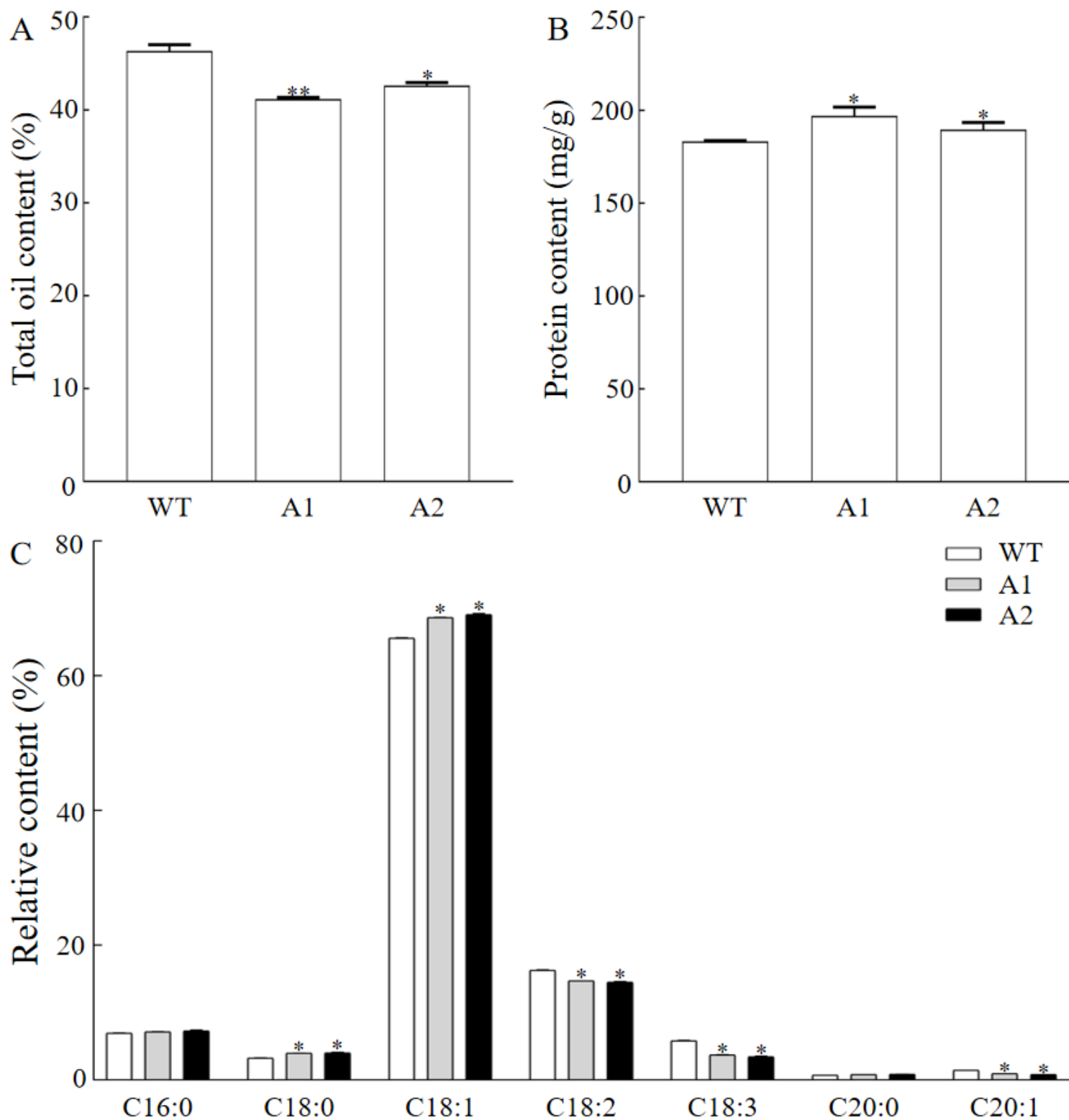


Figure 9

The oil content and fatty acid composition of WT and transgenic *B. napus* lines. (A) Total oil contents of WT, A1, and A2 seeds. (B) The protein contents of WT, A1, and A2 seeds. (C) Fatty acid compositions of WT, A1, and A2 seed oil. C16:0, palmitic acid; C18:0, oleic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; C20:0, arachidic acid; C20:1, cis-11-eicosenoic acid. Each values were displayed as means $\pm$ S.D. of three independent replicates.

Supplementary Files

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