A Moss 2-Oxoglutarate/Fe(II)-Dependent Dioxygenases (2-ODD) Gene of Flavonoids Biosynthesis Positively Regulates Plants Abiotic Stress Tolerance

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Flavonoids, the largest group of polyphenolic secondary metabolites present in all land plants, play essential roles in many biological processes and defense against abiotic stresses. In the flavonoid biosynthesis pathway, flavones synthase I (FNSI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), and anthocyanidin synthase (ANS) all belong to 2-oxoglutarate/Fe(II)-dependent dioxygenases (2-ODDs) family, which catalyzes the critical oxidative reactions to form different flavonoid subgroups. Here, a novel 2-ODD gene was cloned from Antarctic moss Pohlia nutans (Pn2-ODD1) and its functions were investigated both in two model plants, Physcomitrella patens and Arabidopsis thaliana. Heterologous expression of Pn2-ODD1 increased the accumulation of anthocyanins and flavonol in Arabidopsis. Meanwhile, the transgenic P. patens and Arabidopsis with expressing Pn2-ODD1 exhibited enhanced tolerance to salinity and drought stresses, with larger gametophyte sizes, better seed germination, and longer root growth. Heterologous expression of Pn2-ODD1 in Arabidopsis also conferred the tolerance to UV-B radiation and oxidative stress by increasing antioxidant capacity. Therefore, we showed that Pn2-ODD1 participated in the accumulation of anthocyanins and flavonol in transgenic plants, and regulated the tolerance to abiotic stresses in plants, contributing to the adaptation of P. nutans to the polar environment.

Keywords: abiotic stress, Antarctic moss, anthocyanin accumulation, 2-oxoglutarate/Fe(II)-dependent dioxygenases (2-ODDs), flavonoids, flavonol

INTRODUCTION

The 2-oxoglutarate/Fe(II)-dependent dioxygenases (2-ODDs) are non-heme iron-containing soluble proteins, which catalyze the oxidation reactions of diverse substrates, including hydroxylation, demethylation, desaturation, halogenation, and epimerization (Wang et al., 2021c; Wei et al., 2021). 2-ODDs mainly participate in the biosynthesis of various metabolic pathways, such as flavonoids, benzylisoquinoline alkaloids, glucosinolates, tropane alkaloids, and plant...
hormones (Kawai et al., 2014; Wang et al., 2019). Flavonoids are the largest class of specialized metabolites in plants, which have been isolated and identified more than 10,000 species. Despite their diversity, they all share the basic 15-carbon phenylpropanoid core (C6-C3-C6 skeleton structure) (Li C. et al., 2017). Flavonoids biosynthesis starts with the general phenylpropanoid pathway to produce p-coumaroyl-CoA. Then, p-coumaroyl-CoA and malonyl-CoA were catalyzed by chalcone synthase (CHS) and chalcone isomerase (CHI) to form naringenin, a key precursor of flavonoids biosynthesis (Jiang et al., 2016; Busche et al., 2021). Naringenin can be either catalyzed by flavone synthase I and/or II (FNSI or FNSII) to produce flavone, or by flavanone-3-hydroxylase (F3H) to form dihydrouvaemperol (DHK). Consequently, DHK can be converted to flavonols or anthocyanins by flavonol synthase (FLS) or dihydroflavonol reductase (DFR) and anthocyanin synthase (ANS) (Ahn et al., 2015; Wang et al., 2021b). Moreover, the modifications and derivatization of the carbon atoms of the basic skeleton, catalyzed by glycosyltransferase and O-methyltransferases, result in the diverse flavonoid groups in nature, including isoflavones, flavones, flavanones, flavanols, flavonols, and anthocyanins (Farrow and Facchini, 2014). Among the enzymes in the flavonoid biosynthesis pathway, flavones synthase I (FNSI), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), and anthocyanidin synthase (ANS)/leucoanthocyanidin dioxygenase (LDOX) belong to the 2-ODDs family, which requires 2-oxoglutarate, Fe²⁺, and ascorbate as co-factors to catalyze a series of oxidation reactions (Park et al., 2019; Busche et al., 2021).

FNSIs and F3Hs exhibit a relatively limited substrate selectivity, whereas FLSs and ANSs can accept a wide range of flavonoid compounds as possible substrates (Li et al., 2020a). FNSIs from primitive land plants (Plagiochasma appendiculatum, Physcomitrella patens, and Selaginella moellendorfii) displayed FNS and F3H dual activities, catalyzing naringenin into apigenin and DHK (Han et al., 2014; Li et al., 2020a). PeFNSI from Petroselinum crispum converted (2R,3S)-cis-DHK to kaempferol in vitro (Martens et al., 2003). F3Hs share high similarity with FNSIs and convert (2S)-flavanones into dihydroflavonols. FLSs are responsible for flavonol biosynthesis, in competition with DFR in the anthocyanin pathway for their common substrates DHK (Martens et al., 2010). Overexpression of FLSs could lead to the accumulation of flavonol and the reduction of anthocyanin in plant tissues (Luo et al., 2015; Jiang et al., 2020). In vitro, recombinant FLS protein from Oryza sativa not only converted DHK and dihydroquercetin (DHQ) into kaempferol and quercetin, but also catalyzed eriodictyol and naringenin to form DHQ and DHK, exhibiting FLS and F3H activities (Park et al., 2019). FLS from Citrus unshiu also catalyzed the unnatural (2R)-naringenin and natural (2S)-naringenin to yield dihydrokaempferol and kaempferol, respectively (Lukačin et al., 2003). FLSs share identity with ANSs at the polypeptide level and accept (2R,3S,4R)-leucoanthocyanidins as substrate (Turnbull et al., 2004). ANS can react with leucoanthocyanidins, flavanones, and dihydroflavonols, as well as (+)-Catechin, displaying a broad variety of substrate selectivity (Park et al., 2019). Functional defects or silencing of ANS affect the formation of plant color, thus resulting in the colorless or white organs (Rafique et al., 2016).

Flavonoids are extensively distributed in extant land plants, including bryophytes and vascular plants, which is thought to be crucial for plants land colonization and adaptation to terrestrial ecosystems (Jiang et al., 2016; Li et al., 2020a; Piatkowski et al., 2020). During plant evolution, different classes of flavonoids appeared sequentially (Koes et al., 1994). First, chalcones, flavanones, and flavones were found in the basal land plants liverworts due to the existence of functional FNSI, whereas proanthocyanidins were present in lycophytes (ferns). Furthermore, in addition to the generation of dominant flavone, both ferns and gymnosperms began to synthesize flavonols. In P. patens, flavonols were also detected (Wolf et al., 2010). Finally, flavonols and anthocyanins appeared with the emergence of angiosperms, harboring the true F3H genes (Li et al., 2020a). Flavonols participate in UV-B protection, male fertility, and regulating plant growth and development (Hamamouch et al., 2020). Anthocyanins, a water-soluble plant pigments, accountable for red to purple colors, are abundant in many plant tissues in seed plants (Tانaka et al., 2008; Pervaiz et al., 2017), which are involved in many critical biological functions, including attracting pollinators for pollination, providing resistance of herbivory, and absorbing UV-B radiation. (Ahn et al., 2015; Pervaiz et al., 2017). However, the downstream branch pathway of flavonoids biosynthesis in the early terrestrial plant bryophytes is not clear, and whether bryophytes can synthesize anthocyanins is still controversial.

Non-vascular plant liverworts can produce cell wall-localized red flavonoid pigment riccionidins (an auronidin), which is formed by a branch of phenylpropanoid metabolism pathway distinct from anthocyanins biosynthesis (Kunz et al., 1993; Berland et al., 2019). And moss Sphagnum capillifolium had been reported to generate sphagnorubins (Mues, 2000), described as “anthocyanin-like” pigments, which contributes to alleviating abiotic stresses, much like the anthocyanins in seed plants (Albert et al., 2018; Piatkowski et al., 2020). In P. patens, several probable 2-oxoglutarate-dependent dioxygenase genes were from its genome, and 2-ODD1 from P. patens exhibited FNSI/F3H activity and catalyzed naringenin to produce apigenin and dihydrokaempferol (Li et al., 2020a). However, there were no detectable anthocyanin pigments in stressed P. patens by high-performance thin-layer chromatography (Wolf et al., 2010). A phylogenetic analysis revealed that investigated seedless plants’ liverworts, mosses, and ferns possessed no orthologs of ANS, and orthologs representing the complete anthocyanin biosynthetic pathway only existed in the seed plants (Piatkowski et al., 2020). However, some 2-ODD genes from S. moellendorfii and P. patens were also annotated as probable ANS. Furthermore, six anthocyanin compounds including peonidin 3-O-glucoside chloride, peonidin O-hexoside, pelargonidin, malvidin 3-O-glucoside, cyanidin 3-O-galactoside, and cyanidin 3-O-rutinoside were recently confirmed to be present in Antarctic moss Leptobryum pyriforme by a widely targeted metabolomics, and cyanidin 3-O-rutinoside was the significantly up-regulated metabolite with log2(Fold change) 14.68 under ultraviolet-B radiation (Liu et al., 2021).
In Antarctica, extreme aridity, cold, and high UV-B radiation severely restrict the growth of terrestrial plants (Singh et al., 2010). The Antarctic plants have evolved independently for millions of years due to the separation of Antarctica from other continents. Mosses, one of the dominant terrestrial plants in the limited ice-free ground, have evolved a series of special physiological mechanisms to adapt tough environments (Convey et al., 2014; Alavilli et al., 2017). For example, in three East Antarctic mosses, the accumulation of cell wall UV-B-absorbing compounds and red pigments acted as a photoprotective mechanism against UV-B radiation (Waterman et al., 2018). Antarctic moss *Andreaea regularis* displayed high levels of carotenoids and UV-B screening pigments in foliage in response to UV-B exposure (Newsham, 2003). Bioflavonoids extracted from Antarctic mosses *Ceratodon purpureus* exhibited UV screening and antioxidant activity, which may be accountable for the high resistance to UV-B radiation (Waterman et al., 2017). The transcriptome profiling of Antarctic moss *Pohlia nutans* under UV-B treatment indicated that the antioxidant system, DNA-repairing system, and flavonoids biosynthesis pathway contributed to the adaptation and survival of moss to UV-B radiation (Li et al., 2019). Antarctic moss *Sanionia uncinata* exhibited tolerance to desiccation due to the activation of protective mechanisms that are involved in increased activity of antioxidant enzymes, accumulation of osmotic adjustment compounds like proline, glycine betaine, and dehydrins proteins (Pizarro et al., 2019). In this study, we isolated a 2-ODD gene from the Antarctic moss *P. nutans* (*Pn2-ODD1*) and investigated its functions in *P. patens* and *Arabidopsis*. These results showed that heterologous expression of *Pn2-ODD1* promoted the accumulation of anthocyanins and flavonol in transgenic plants, and conferred plant tolerance to salt, drought, and UV-B stress by increasing antioxidant capacity.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis thaliana* (Col-0) was used as the WT plant and for the generation of transgenic lines. *Arabidopsis* was grown in a greenhouse at 22°C and 60% relative humidity, with 8-16 h light/16-8 h dark (light intensity, 100 µmol·m⁻²·s⁻¹) for vegetative growth. After about 4 weeks, they were cultured in a growth chamber with 16-h light for subsequent reproductive maturation. *Physcomitrella patens* were cultivated on BCD solid medium at 25°C, in 16-h light/8-h dark photoperiod (60 µmol·m⁻²·s⁻¹). For the production of protonema cells, *P. patens* were homogenized by a polytron homogenizer and cultivated on agar medium (BCD, supplemented with 5 mM diammonium tartrate) overlaid cellophane for convenient subculture.

**Bioinformatics Analysis**

The sequence of *Pn2-ODD1* gene was obtained from the transcriptome of Antarctic moss *Pohlia nutans* using HMMER program (Li et al., 2019). Subsequently, the multiple sequence alignments of *Pn2-ODD1* and other plant 2-ODDs were performed using DNAMAN software. The neighbor-joining method was used to construct the phylogenetic tree using MEGA 5.0 software.

**RNA Isolation and Quantitative Real-Time PCR Analysis**

Total RNA was extracted from *Arabidopsis* and *P. patens* by TRIzol reagent and CTAB method. cDNA was synthesized from 2 µg RNA by 5× All-In-One RT MasterMix (abm, Canada), according to the manufacturer's instructions. Then, quantitative real-time PCR analysis was performed in an LC480 Thermal Cycler instrument by using the SYBR qPCR Master Mix (Nuweizan, Nanjing, China). *AtTubulin* and *PpActin* were used as an internal control. The expression levels of genes were presented by the comparative 2⁻ΔΔCt method (Livak and Schmittgen, 2001). All used primers were listed in Supplementary Table 1.

**Plasmid Constructions and Plant Transformation**

The full length of *Pn2-ODD1* was amplified by PCR from cDNA of *Pohlia nutans* with the specific primers. And the PCR fragment was inserted into the Smal restriction site of the pTFH15.3 vector by one-step cloning (Nuweizan, Nanjing, China). Then, the constructed *Pn2-ODD1*-pTFH15.3 plasmid was linearized by NotI to introduce into *P. patens* protoplasts by PEG-mediated DNA uptake, with a slight modification (Cove et al., 2009). Different concentrations of antibiotic G418 (25, 50, or 100 µg·mL⁻¹) were used to select surviving transformants. Finally, stable transformants were screened by PCR amplification for phenotypic analysis.

For expressed-*Pn2-ODD1* Arabidopsis, *Pn2-ODD1* gene was cloned into the pRI101 vector to obtain *Pn2-ODD1*-pRI101. The recombinant construct was then introduced into *Arabidopsis* (Col-0) plants via Agrobacterium-mediated infiltration method (Zhang et al., 2006). Positive transgenic seedlings were screened on 1/2 MS medium supplemented with 50 µg·mL⁻¹ kanamycin. Two independent T3 *Pn2-ODD1* transgenic lines were obtained for subsequent analysis.

**Measurement of Anthocyanin and Flavonols Contents**

For the observation of anthocyanin enrichment in 5-day-old *Arabidopsis* seedlings, the seeds were cultivated on 1/2 MS agar medium, with 24-h light for 5 days at 22°C (Li P. et al., 2017). For the accumulation of anthocyanins in 17-day-old seedlings, sterilized seeds were germinated on 1/2 MS solid medium containing 3% (w/v) sucrose in a culture room with 16-h light for 14 days, then transferred on 1/2 MS medium supplemented with 12% (w/v) sucrose for another 3 days (Yonekura-Sakakibara et al., 2012). The purple coloration of seedlings was recorded, and plant samples were collected for the spectrophotometry measurement of anthocyanin levels and the analysis of flavonoids or anthocyanins metabolomics.

Anthocyanin extraction from *Arabidopsis* was carried out as described in Xu et al. (2017). Briefly, homogenized samples were incubated with methanol-HCl (1%, w/v) at 4°C for 24 h in...
the dark. Then, the extracts were mixed with chloroform and distilled water to remove chlorophyll. After centrifugation at 12,000 g for 15 min, the supernatant was collected and measured the absorbance at 657 and 530 nm. The anthocyanin level was expressed as (A530-0.25A657)/fresh weight (g).

The total flavonols were extracted from Arabidopsis according to the method of Wang et al. (2020), with a slight modification. 0.2 g of fresh samples were ground with liquid nitrogen and extracted using 50% methanol. After centrifugation at 13,000 g for 15 min, the supernatant was transferred into a new tube, followed by adding an equal volume of 2 N HCl and hydrolyzing at 70°C for 40 min. Then, 100% methanol was added to prevent the degradation of the aglycones. The mixture was centrifuged for 15 min at 13,000 g to collect the supernatants for HPLC analysis (Shimadzu LC-20A, Japan), equipped with an Agilent C18 column (4.6 x 250 mm, 5 µm). The mobile phase was 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B). The linear gradient conditions were as follows: 0 min, 5% B; 30 min, 55% B; 45 min, 65% B; 50 min, 100% B; 52-60 min, 5% B, at a flow rate of 1 mL/min. The column temperature was set at 30°C, and the flavonols were detected at 365 nm (Park et al., 2019).

**Targeted Metabolomics Analysis**

The flavonoids and anthocyanins metabolomics analyses in Arabidopsis plants were performed by Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China). In general, the freeze-dried sample was homogenized into powder. For widely targeted flavonoids metabolomics, 0.1 g powder was dissolved with 70% aqueous methanol at 4°C overnight. After centrifugation, the filtered supernatant was analyzed using LC-MS/MS (UPLC; Shim-pack UFLC SHIMADZU CBM30A system; MS, Applied Biosystems 6500 Q TRAP). Based on a self-built database and a public metabolite database, the metabolites were qualitatively identified by secondary spectral properties. Differential metabolites with VIP (variable importance in the project) ≥ 1, fold change ≥ 1.2, or fold change ≤ 0.83 were filtered as significantly changed metabolites.

For targeted anthocyanins metabolomics analysis, 50 mg homogenized powder was incubated with an extraction solvent containing 0.5 mL methanol/water/hydrochloric acid (500:500:1, V/V/V) by vortex and ultrasound for 5 min. After that, the sample was centrifuged at 12,000 g at 4°C for 3 min. Finally, the supernatants were collected and filtrated to analyze by Ultra Performance Liquid Chromatography and ESItriple quadrupole-linear ion trap mass spectrometer (QTRAP). For the absolute quantitative analysis of anthocyanins, commercial standard substances were diluted to different concentrations to obtain the corresponding mass spectrum peak intensity. Then, the absolute contents of anthocyanins in extracted samples were calculated by the standard curves of different substances. The differential metabolites were selected according to the P values (≤ 0.05) and fold change (≥ 1.2 or ≤ 0.83).

**Plant Stress Treatments**

For stress treatments of transgenic P. paten, the stem tips with the same size of transgenic Pn2-ODD1 and WT gametophytes were placed on a BCD medium supplemented with different concentrations of NaCl and D-Mannitol for 5 weeks at 25°C. The visual phenotypes were photographed, and the colony diameters were measured.

For drought stress assay, 3-week-old Arabidopsis seedlings grown in soil containers were subjected to drought stress by depriving water for 21 days, and then rewatered for 3 days to calculate the survival rates. For the seed germination of Arabidopsis, the sterilized seeds were sown on the 1/2 MS solid medium with or without NaCl, D-mannitol, H2O2, and 3-amino-1,2,4-triazole (3-AT, a CAT inhibitor, triggering the accumulation of H2O2). Then plates were placed in the dark at 4°C for 2 days and then cultured in a greenhouse at 22°C for 4–8 days. The germination rate was represented by counting the proportion of cotyledon greening. For root length assay, seedlings were vertically grown on a 1/2 MS medium containing 75 or 100 mM NaCl, 0.2 or 0.3 M D-mannitol, and 0.75 or 1 mM H2O2. After 5–9 days, the root length was calculated using Image J software. For the expression analysis of genes under stress treatment, 2-week-old Arabidopsis were sprayed with 200 mM NaCl, 16% PEG, or 20 mM H2O2 for 2 h, and all plant samples were harvested and frozen at −80°C until use.

For UV-B treatment, 3-week-old WT and transgenic Pn2-ODD1 Arabidopsis seedlings were treated for 12 h with 0.25 mW·cm−2 UV-B intensity. After being recovered for 3 days, the seedlings were collected and immediately frozen in liquid nitrogen and stored at −80°C for further analysis. Arabidopsis cultivated under the normal light conditions was used as the control.

**Chlorophyll Content Quantification**

For the measurement of chlorophyll content, homogenized samples were extracted with 80% acetone at 4°C in the dark. After centrifugation, the supernatant was collected to detect the absorbance at 663 and 645 nm by spectrophotometry analysis. Total chlorophyll content was calculated using the following formula: (8.05 × A663 + 20.29 × A645) × mL acetone mg−1 fresh weight (Porra et al., 1989).

**3,3′-Diaminobenzidine (DAB) and Nitroblue tetrazolium Blue Chloride (NBT) Staining**

DAB and NBT staining were used to detect the accumulation of hydrogen peroxide (H2O2) and superoxide anion (O2−) as described previously (Wang et al., 2015), with a slight modification. Whole seedlings were incubated with 1 mg·mL−1 DAB solution or 1 mg·mL−1 NBT solution in 50 mM potassium phosphate buffer (pH7.8) for 12 h in the dark. Then, chlorophyll was completely removed by decolorization with a bleaching solution (ethanol:acetic acid:glycerol = 3:1:1) in a boiling water bath.

**Statistical Analysis**

All experiments were performed at least three times for biological replicates. All data were expressed as mean ± standard deviation (SD).
RESULTS

Characterization and Sequence Analysis of Pn2-ODD1 Gene

The full-length cDNA sequence of Pn2-ODD1 was amplified by PCR, which contains an open reading frame of 1,086 bp, encoding a 403 kDa-polyepitope with 361 amino acids and a predicted isoelectric point of 5.53. Multiple alignment analysis showed that Pn2-ODD1 shared only 35.3% similarity with the 2-ODD1 from P. paten and 36.7% similarity with FNSI from P. patens, whereas about 25.0% identity with other known species 2-ODDs. However, Pn2-ODD1 contained the conserved domains: Fe23 binding site HxDxH (His228, Asp230, and His285) and the 2-oxoglutarate (2-OG) binding domain RxS (Tyr213, Arg295, and Ser297) (Figure 1). Phylogenetic analysis showed that in the members of a 2-ODDs family in flavonoids biosynthesis pathway, ANSs, and FLSs belonged to one subclade, while Apiaceae FNSIs and F3Hs constituted another independent branch. Pn2-ODD1 clustered with FLSs and ANSs branch and was closely related to S. moellendorffii and P. patens 2-ODDs proteins (Figure 2).

Pn2-ODD1 Contributed to the Accumulation of Anthocyanin and Flavonols in Transgenic Arabidopsis

Arabidopsis is a model organism in the plant kingdom, due to the easily quantitative stress-resistance indicators, clear flavonoids biosynthesis pathway, and regulatory networks, which is beneficial to promote the substantial development and application of genetic resources in higher plants. To explore the biological function of Pn2-ODD1, two independently expressed Pn2-ODD1 Arabidopsis were identified by genomic PCR (Supplementary Figure 1). Constant light can induce the accumulation of flavonoids in plants. After the sterilized seeds were sown on the 1/2 MS medium and placed vertically with the constant light for 5 days, both WT and Pn2-ODD1-expressed Arabidopsis displayed the accumulation of flavonoids, with the purple hypocotyls. However, ATOE lines accumulated higher total flavonoids and anthocyanin levels (spectrophotometry analysis), with the deeper hypocotyls, which were 1.30- and 1.35-fold in contrast with WT plants, respectively (Figure 3A–D). The flavonoid metabolomics of 5-day-old WT and ATOE lines were detected based on UPLC-MS/MS method. A total of 156 metabolites were identified, including 59 flavones, 71 flavonols, two isoflavones, 10 flavanols, three chalcones, three anthocyanins, and eight flavone C-glycosides (Figure 3E). According to VIP value ≥ 1 and fold-change threshold ≥ 1.2 or ≤ 0.83, 48 differential metabolites (36 up-regulate and 12 down-regulate) were screened (Figure 3F and Supplementary Table 2). Among them, flavonols were the most abundant compounds (16 up-regulated and six down-regulated) (Figure 3G). Notably, three detected anthocyanins were all up-regulated. KEGG classification analysis showed that the differential metabolites dominantly focused on the flavonoid and anthocyanin biosynthesis metabolism (Figure 3H).

Also, when cultivated on 1/2 MS medium containing sucrose, transgenic Pn2-ODD1 Arabidopsis exhibited greater anthocyanin accumulation, with deeper purple leaves, which was about 1.60-fold in contrast with WT plants (Figures 4A–C). Meanwhile, the expression levels of anthocyanin synthesis pathway genes AtPAL, AtDFR, and AtUFGT were increased in AtOE lines (Figure 4D). To further determine the difference in anthocyanin metabolism profiles, we performed the targeted anthocyanin metabolomics of AtOE lines and WT plants induced by sucrose. As shown in Figure 4E, the OE1 line had a 17.6% increase in the levels of total anthocyanins than that of the WT plants, which was consistent with the result measured by spectrophotometry analysis (anthocyanins at A530 nm). Totally, 31 anthocyanin metabolites were detected, including cyanidin (14), delphinidin (5), petunidin (3), pelargonidin (3), and peonidin (6). Based on a fold-change threshold ≥ 1.2 or ≤ 0.83, 7 up-regulated differential metabolites were identified (Figure 4F). Among them, cyanidin-3-O-(6-O-malonyl-beta-D-glucoside) and peonidin-3,5-O-diglucoside were the most significantly differential metabolites with log2(fold change), which were 1.65 and 1.56 folds compared with WT plants, respectively (Figure 4G). Then, the content of flavonol (including quercetin and kaempferol) in the WT plants and expressed-Pn2-ODD1 Arabidopsis grown with sucrose was analyzed using HPLC. The results showed that there was no significant difference in flavonol components (mainly quercetin and kaempferol) between WT plants and ATOE lines. However, the content of total flavonols in transgenic Arabidopsis was increased by 50.0%, in contrast with WT plants (Figures 4H, I). Among them, the levels of quercetin and kaempferol were significantly enhanced, which were about 65.0% and 50.0% higher than that of the WT plants, respectively (Figure 4J). These results suggested that the heterologous expression of Pn2-ODD1 resulted in the enrichment of anthocyanin and flavonols in Arabidopsis.

Pn2-ODD1 Conferred the Tolerance to Salt Stress in Transgenic Physcomitrella patens and Arabidopsis

P. patens has become an important model plant for functional gene research due to its rapid growth cycle and mature genetic transformation methods (He et al., 2019; Rensing et al., 2020). Four independent transgenic P. patens (#1, #4, #7 and #8) were obtained and confirmed by genomic PCR analysis to further determine the function of Pn2-ODD1 in response to abiotic stress (Supplementary Figure 1). The stem tips with the same size of transgenic lines and WT plants were grown on BCD solid medium containing 100, 125, and 150 mM NaCl. Under normal medium, there was no obvious difference in growth performance between expressed-Pn2-ODD1 P. patens and WT plants. However, in the presence of NaCl, the transgenic P. patens displayed larger gametophytes than the WT plants. On 100 mM NaCl medium, the clone size of WT plants was 5.03 mm, whereas those of transgenic P. patens were 7.39, 7.50, 7.67, and 7.22 mm. On 125 or 150 mM NaCl, the diameter of the gametophyte of transgenic P. patens was still larger than that of the wild type, which was about an increase of 1.35- and 1.50-fold, respectively (Figures 5A, B). In Arabidopsis, no detectable differences in germination rate and root length were observed between WT and ATOE lines in the absence of
FIGURE 1 | Pn2-ODD1 shared homology with other known species 2-ODDs. The amino acid sequence alignment of Pn2-ODD1 with 2-ODDs protein from other species. Red frames indicated ferrous iron binding site HXD X55H and 2-oxoglutarate (2-ODD) binding site RXS. Red asterisks (*) indicated the conserved amino acid residues of 2OG-FeII_Oxy conserved domain.

ZmFNSI, AtDMR6, PnFNSI, PfFNSI, AtF3H, DcF3H, GbF3H, TaF3H, AtFSL1, GmFSL, OsANS, ZmAANS, Pp2-ODD1, Pn2-ODD1.
NaCl. On 1/2 MS mediums supplemented with 75 mM NaCl, the germination rate of expressed-\textit{Pn2-ODD1} plants was 78.0% and 81.7%, which was about 15.0% higher than that of the WT plants. Similarly, at 100 mM NaCl, \textit{AtOE} lines also displayed better germination, with an increase of 20.0% in comparison with WT plants (\textit{Figures 5C,D}). For root length assays, the early root length in the transgenic \textit{Pn2-ODD1 Arabidopsis} was markedly more vigorous, which was about 1.30-fold or 1.55-fold longer...
FIGURE 3 | Prn2-ODD1 increased the levels of flavonoids in 5-day-old Arabidopsis. (A) Anthocyanin accumulation visualized by the purple coloration in 5-day-old seedlings with constant light. (B) Anthocyanin extraction solutions for WT and AtOE lines. (C,D) Contents of total flavonoids and anthocyanins in 5-day-old seedlings detected by spectrophotometry analysis. (E) Number of different classes of flavonoids in 5-day-old WT and transgenic Arabidopsis detected by the flavonoids.
FIGURE 3 | Volcano plot of samples. The volcano plot showed the levels of flavonoids metabolites and the statistical significance. Each point represents a metabolite. Horizontal ordinate indicates the fold change of flavonoids metabolites between WT and OE1, while VIP value means a significant difference in statistical analysis. (G) Statistical analysis of the classes of differential flavonoid metabolites. (a-f) Flavones, flavonols, anthocyanins, flavanols, chalcones, and flavone C-glycosides. (H) Differential metabolites KEGG classification. Asterisk (*) represents a significant difference between the WT plants and AtOE lines (Student's t-test, *P < 0.05, **P < 0.01).

than that of the WT plants at 75 mM NaCl or 100 mM NaCl (Figures 5E,F).

To further investigate the possible molecular mechanism of increased tolerance to salt stress in transgenic Pn2-ODD1 lines, the transcript levels of stress-responsive genes were analyzed by qRT-PCR. We found that the expression patterns of salt tolerance genes PpSHPI and PpSHP2, stress responsible genes PpCOR TMC-AP3 and PpCOR47 were markedly up-regulated in transgenic P. patens, in contrast with WT plants (Figure 5G). Meanwhile, the expression patterns of salt tolerance genes AtHKT1, AtNHX1, AtSOS3, and AtP5CS1, and antioxidiant enzymes gene AtCu/Zn-SOD3 were markedly increased in AtOE lines, while ROS generation gene AtRibohD was down-regulated in expressed-Pn2-ODD1 Arabidopsis (Figure 5H). Therefore, these results suggested that enhanced salt tolerance by Pn2-ODD1 might be correlated with upregulating several stress-related genes.

**Pn2-ODD1 Enhanced the Resistance to Drought Stress in Transgenic Plants**

Salt tolerance is usually associated with osmotic resistance. Thus, the growth performance of WT and expressed-Pn2-ODD1 plants under drought stress was analyzed. As shown in Figures 6A,B, the heterologous expression of Pn2-ODD1 significantly increased the resistance to D-mannitol stress in P. patens. On 0.3 M D-mannitol medium, the clone size of expressed-Pn2-ODD1 P. patens was 8.32, 8.73, 8.44, and 8.31 mm, which was about 40.0% higher than that of WT plants (6.11 mm), following the formation of protonema. When exposed to 0.4 M D-mannitol, the diameter of the protonema of transgenic P. patens was about 6.33 mm, which was 1.5-fold in contrast with WT plants. In Arabidopsis, 3-week-old seedlings with the same growth were treated with water withdrawal for 21 days. As shown in Figure 6G, AtOE lines exhibited better performance with a 65.0% survival rate, whereas WT plants were severely damaged and became wilted, with a 32.5% survival rate (Figure 6H). The sterilized seeds were sown on 1/2 MS medium supplemented with different concentrations of D-mannitol to observe the germination and root growth. On 0.2 M D-mannitol treatment, transgenic Pn2-ODD1 lines displayed 61.6–68.3% germination rates, which was about 20.0% higher than WT plants (Figures 6C,D). The root resistance assays demonstrated that AtOE lines exhibited longer root length, which was about 1.20-fold at 0.2 M D-mannitol and 1.60-fold at 0.3 M D-mannitol, in contrast with WT plants (Figures 6E,F). Drought stress can result in the generation of reactive oxygen species (ROS). The contents of H$_2$O$_2$ in AtOE lines were significantly lower than those of WT plants after 16% PEG treatment (Figure 6I). Antioxidant enzymes, such as CAT and SOD, participate in the reduction process of ROS (Choudhury et al., 2013). qRT-PCR analysis demonstrated that the expression levels of ROS-scavenging related gene AtCAT1, AtFeSOD1, AtCu/Zn-SOD3, and proline biosynthesis-related gene AtP5CS1 were markedly increased in transgenic Pn2-ODD1 Arabidopsis (Figure 6J). These results demonstrated that Pn2-ODD1 contributed to enhanced resistance to drought stress by increasing ROS clearance capacity in transgenic plants.

**Heterologous Expression of Pn2-ODD1 Increased Arabidopsis Tolerance to UV-B Radiation and Oxidative Stress**

To investigate whether Pn2-ODD1 could enhance the plant tolerance to UV-B stress, 3-week-old Arabidopsis were subjected to UV-B radiation with 0.25 mW·cm$^{-2}$ UV-B intensity for 12 h and recovered for 3 days. When compared with WT plants, AtOE lines displayed better growth performance, with greener leaves and less damage (Figure 7A). The chlorophyll degradation ratio of transgenic Pn2-ODD1 Arabidopsis was lower than that of the WT plants under UV-B treatment (Figure 7B). DAB staining suggested that AtOE lines accumulated less endogenous H$_2$O$_2$ levels, in contrast with WT plants (Figure 7C). Furthermore, the transcript patterns of ROS-scavenging genes AtCu/Zn-SOD1, AtCu/Zn-SOD2, AtCu/Zn-SOD3, AtCAT1, and AtCAT3 were up-regulated in expressed-Pn2-ODD1 Arabidopsis (Figure 7D).

In general, abiotic stresses lead to a build-up of ROS, which results in oxidative stress (Cruz de Carvalho, 2008; He et al., 2018). H$_2$O$_2$ and 3-amino-1,2,4-triazole (3-AT, a CAT inhibitor, triggering the accumulation of H$_2$O$_2$ were used to simulate exogenous and endogenous oxidative stress for young seedlings, respectively. The results showed that heterologous expression of Pn2-ODD1 conferred the enhanced tolerance to exogenous and endogenous oxidative stress in Arabidopsis. In the presence of 0.75 mM H$_2$O$_2$, the root length of AtOE lines was 1.86 and 1.94 cm, which was an increase of 30.5%, compared with the WT plants (Figures 8A,B). Furthermore, the lateral root numbers of AtOE lines were markedly higher than that of the WT plants, which were about 1.60-fold longer at 0.75 mM H$_2$O$_2$, whereas 1.35-fold longer at 1.0 mM H$_2$O$_2$ (Figure 8C). qRT-PCR analysis demonstrated that the expression levels of AtFeSOD1, AtFeSOD2, and AtCAT1, encoding ROS-scavenging enzymes, were significantly up-regulated in expressed-Pn2-ODD1 Arabidopsis (Figure 8D). Under endogenous oxidative stress, AtOE lines displayed reduced sensitivity to 3-AT, with greener leaves and lower H$_2$O$_2$ and O$_2^-$ levels, in contrast with WT plants (Figures 8E–G). Previous reports had demonstrated that ROS can trigger the accumulation of anthocyanins (Xu et al., 2017). Consistent with the results, under the treatment of 3-AT for 7 days, the levels of total flavonoid and anthocyanins in AtOE lines were markedly increased by
Figure 4 | \textit{Pn2-ODD1} enhanced the accumulation of anthocyanins and flavonols in 17-day-old \textit{Arabidopsis} seedlings induced by sucrose. (A) Anthocyanin accumulation visualized by the purple coloration in 17-day-old seedlings induced by sucrose (cultured on a medium containing 3% sucrose for 14 days, then transferred to 12% sucrose medium for 3 days). (B, C) Anthocyanin extraction solutions and anthocyanin contents (spectrophotometry analysis). (D) Expression levels of genes in anthocyanin biosynthesis pathway in WT and AtOE lines induced by sucrose. (E) The absolute quantitative analyses of total anthocyanins in 17-day-old \textit{Arabidopsis} seedlings induced by sucrose detected by targeted metabolomics strategy. (F) Volcano plot of samples. It showed the levels of anthocyanins metabolites and the statistical significance of the difference. Each point represents a metabolite. Horizontal ordinate represents the fold change of anthocyanins metabolites between two samples, while \( p \)-value means significant difference in statistical analysis. (G) 7 up-regulated anthocyanins metabolites identified in the transgenic \textit{Pn2-ODD1} \textit{Arabidopsis}. 1–7: Cyanidin-3,5-O-diglucoside, cyanidin-3-O-rutinoside, cyanidin-3-O-(6-O-malonyl-beta-D-glucoside),
FIGURE 4 | cyanidin-3-O-5-O-(6-O-coumaryl)-diglucoside, delphinidin-3-O-rutinoside, peonidin-3,5-O-diglucoside, and peonidin-3-O-(6-O-p-coumaryl)-glucoside. (H) HPLC profiles of flavonols standards (myricetin, quercetin, and kaempferol) and the flavonols extracts from WT and expressed-Pn2-ODD1 Arabidopsis (365 nm). (I,J) Contents of total flavonol, quercetin, and kaempferol in WT and AtOE lines induced by sucrose. Asterisk (*) represents a significant difference between the WT plants and AtOE lines (Student’s t-test, *P < 0.05, **P < 0.01, ***P < 0.001).

FIGURE 5 | Heterologous expression of Pn2-ODD1 enhanced plant resistance to salt stress. (A) The transgenic P. patens displayed larger gametophytes than the WT plants under NaCl treatment. (B) Statistical analysis of gametophyte size as shown in (A). (C,E) AtOE lines exhibited higher germination rates and longer root length in contrast with WT plants under salt stress. (D,F) Statistical analysis of the seed germination rates and root length as shown in (C,E). (G,H) Expression patterns of stress-responsive genes in Pn2-ODD1 transgenic P. patens and Arabidopsis measured by qRT-PCR. Asterisk (*) represents a significant difference between the WT plants and AtOE lines (Student’s t-test, *P < 0.05, **P < 0.01).

15.0% and 40.0%, in contrast with WT plants, respectively (Figures 8H,I). Thus, these results illustrated that Pn2-ODD1 improved the resistance to oxidative stress by increasing ROS scavenger and anthocyanins accumulation.

DISCUSSION

2-Oxoglutarate/Fe(II)-dependent dioxygenases (2-ODDs), non-heme iron-containing soluble proteins, participate in the synthesis of important plant hormones and diverse secondary metabolites (Farrow and Facchini, 2014; Wei et al., 2021). Flavonoids, the natural products present in plants, possess enormous chemical diversity due to the organization and modifications of the three-ring structure by 2-ODDs, cytochrome P450-dependent oxygenases (CYPs), glycosyltransferase, and O-methyltransferases (Wang et al., 2019). In flavonoids biosynthesis, the key enzymes of downstream branch pathways FNSI, F3H, FLS, and ANS all belong to the 2-ODDs family, which is involved in the synthesis of key flavonoids compounds, such as flavones, flavonols, and anthocyanins. During the plant land colonization, the acquisition of flavonoids is considered to be an important adaptation for defense against the adverse environmental stresses faced with the transition to a non-aquatic lifestyle (Albert et al., 2018). Bryophytes are thought to be the
FIGURE 6 | Pn2-ODD1 increased the resistance to drought stress in transgenic P. patens and Arabidopsis. (A) The diameter of transgenic P. patens gametophytes was markedly larger than that of the WT plants under drought stress conditions. (B) Statistical analysis of gametophyte size as shown in (A). (C,E) Expressed-Pn2-ODD1 Arabidopsis exhibited higher germination rates and longer root length compared with WT plants under osmosis stress. (D,F) Statistical analysis of the seed germination rates and root length as shown in (C,E). (G) Three-week-old Arabidopsis seedlings grown in soil were withheld water for 21 days, and then rewatered for 3 days. (H) Statistical analysis of the survival rate, as shown in G. (I) H$_2$O$_2$ content in Arabidopsis under 16% PEG treatment. (J) Gene expression levels of ROS-scavenging related genes in Arabidopsis analyzed by qRT-PCR. Asterisk (*) indicates a significant difference between the WT plants and AtOE lines (Student’s t-test, *P < 0.05, **P < 0.01).

oldest terrestrial plants, which exhibit the simple morphological organization, but the complex chemical diversification (Wellman et al., 2003; Asakawa et al., 2013; Ludwiczuk and Asakawa, 2019). Although there are several reports on the function of enzymes (4CL, CHS, CHI, and FNSI) in the upstream flavonoids pathway in bryophytes (Han et al., 2014; Gao et al., 2015; Yu et al., 2015; Cheng et al., 2018), the enzymes responsible for the downstream branch pathways of flavanone in bryophytes remain unclear. In particular, whether anthocyanin and anthocyanin synthase are present in bryophytes is still controversial. In this study, a Pn2-ODD1 gene from Antarctic moss Pohlia nutans and its roles in flavonoids metabolism and abiotic stresses were studied. The amino acid sequence of Pn2-ODD1 shared only 35.3% identity with 2-ODD1 of P. patens and 36.8% identity with PnFNSI, whereas below 30.0% sequence identity with 2-ODDs from other plants. But Pn2-ODD1 possessed the conserved Fe$^{2+}$ binding domains and 2-oxoglutarate (2-OG) binding domains (Figure 1), which was consistent with other members of the 2OG-FeII_Oxy dioxygenase family (Reddy et al., 2007; Han et al., 2014; Zhang et al., 2016; Wang et al., 2020). Phylogenetic analysis demonstrated that Pn2-ODD1 had a closer relationship with ANSs and FLSs, which clustered with the 2-ODDs from P. patens and S. moellendorffii (Figure 2).

2-ODDs family of flavonoids synthesis pathway participate in catalyzing the oxidation of the flavonoid “C ring” to form different flavonoid subclasses. F3Hs and FNSIs can both react with flavanones, while FNSIs in primitive land plants also displayed F3H activity (Li et al., 2020a). Both FLSs and ANSs...
FIGURE 7  Heterologous expression of Pn2-ODD1 enhanced the Arabidopsis resistance to UV-B radiation. (A) Three-week-old Arabidopsis were treated with 0.25 mW·cm⁻² UV-B intensity for 12 h and then recovered for 3 days. (B) Chlorophyll degradation ratio of WT plants and ATOE lines. (C) Levels of hydrogen peroxide (H₂O₂) measured by 3,3’-diaminobenzidine (DAB) staining under UV-B radiation. (D) Gene expression patterns of antioxidant enzymes under UV-B treatment. Asterisk (*) represents a significant difference between the WT plants and ATOE lines (Student’s t-test, *P < 0.05, **P < 0.01).
the non-transformed plants (Holton et al., 1993; Nielsen et al., 2002). Also, overexpression of ANS from *Theobroma cacao* in tobacco led to flower petal color changes, which was consistent with an increased level of anthocyanins in flower petals (Liu et al., 2013). Previously, transgenic rice with ANS accumulated the increased anthocyanins and flavonols, and the reduced proanthocyanin levels, suggesting that rice ANS may be a multifunctional dioxygenase (Reddy et al., 2007). Overexpressed-*RtLDOX2* *Arabidopsis* exhibited the enhanced anthocyanin and flavonol contents, possibly due to the versatility and mutual substitutability of *RtLDOX2* in anthocyanin and flavonol biosynthesis (Li et al., 2021). In *Arabidopsis*, *fls1-2* mutant seedlings displayed the decreased flavonol glycosides levels and accumulated the glycosylated...
Drought stress can lead to a decline in quantity and quality of crop production (Naing et al., 2018). The biosynthesis of flavonoids in plants can be up-regulated when subjected to drought stress (Ma et al., 2014). Metabolome and transcriptome profiling in Arabidopsis showed that the overaccumulation of flavonoids was involved in enhanced tolerance to drought and oxidative stress in MYB overexpressers, transparent testa4 (tt4), and WT plants (Nakabayashi et al., 2014). Overexpression of PnFNS1 contributed to the increased drought resistance by enhancing the antioxidant capacity in Arabidopsis (Wang et al., 2020). Overexpression of RtLDOX2 from Reaumuria trigyna conferred enhanced tolerance to drought, UV-B, and salt stress in Arabidopsis by promoting the levels of anthocyanins and flavonols (Li et al., 2021). Here, heterologous expression of Pn2-ODD1 contributed to the improved tolerance to drought stress in plants (Figure 6). The generation of ROS can be caused by drought stress. Reducing ROS enrichment in transgenic plants usually increased the resistance to drought stress (Choudhury et al., 2013). Transgenic tobacco overexpressing F3H from Lycium chinense displayed enhanced resistance to drought stress, improving the antioxidant system (Song et al., 2016). In this study, after 16% PEG treatment, Pn2-ODD1 decreased the levels of H$_2$O$_2$ in Arabidopsis (Figure 6I). Superoxide dismutase (SOD) and catalase (CAT) are important antioxidant enzymes, which participate in the process of scavenging ROS in plants. SOD catalyzes the conversion of superoxide anions O$_2^-_i$ into H$_2$O$_2$ and O$_2$, and CAT converts H$_2$O$_2$ to produce H$_2$O and O$_2$ (Sharma et al., 2012). qRT-PCR analysis showed that Pn2-ODD1 markedly up-regulated the expression patterns of AtCAT1, AtFeSOD1, and AtCu/Zn-SOD3 under 16% PEG treatment (Figure 6J).

Due to lower stratospheric ozone levels, plants are exposed to increased solar UV-B irradiation, which leads to oxidative damage to DNA, proteins, and lipids (Wolf et al., 2010). Flavonoids compounds, as a non-enzymatic antioxidant system, exhibit high antioxidant activity to protect plants from UV-B and oxidative damage (Buer et al., 2010). In Arabidopsis, anthocyanin-deficient mutants generated more ROS in vivo, accompanied by reduced antioxidant ability (Xu et al., 2017). Overexpression of rice ANS in rice mutant Nootripathu (NP) exhibited enhanced antioxidant activity by promoting the accumulation of anthocyanin and other flavonoids (Reddy et al., 2007). Overexpressing ZmFNSII or ZmFNSII Arabidopsis displayed less UV-B-induced damage due to the accumulation of apigenin, when compared with WT plants (Righini et al., 2019). In our study, heterologous expression of Pn2-ODD1 conferred the tolerance to UV-B stress, exhibiting lower ROS levels and increased expression of antioxidant enzymes genes in Arabidopsis (Figure 7). Consistent with these results, we observed that heterologous expression of Pn2-ODD1 in Arabidopsis conferred the tolerance to exogenous oxidative stress with longer primary roots and up-regulated the transcript levels of ROS scavenging gene, AtFeSOD1, AtFeSOD2, and AtCAT1 (Figures 8A–D). ROS can induce the production of anthocyanins, and anthocyanin-deficient mutants accumulated more endogenous ROS and were hypersensitive to ROS (Xu et al., 2017). 3-AT, a CAT inhibitor, was used to stimulate endogenous oxidative stress. AtOE lines seedlings displayed lower sensitivity to ROS, less
ROS accumulation, and markedly increased total flavonoids and anthocyanin levels, when exposed to 3-AT treatment (Figures 8E–1).

In conclusion, our results provided some evidence that a Pn2-ODD1 gene from P. nutans increased the accumulation of anthocyanin and flavanol in transgenic plants. Also, heterologous expression of Pn2-ODD1 conferred the plant resistance to salinity, drought, and UV-B stress, which may play a key role in the adaptation of P. nutans to the polar environment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PZ and SL designed and supervised the experiments. HW conducted the experiments and wrote the manuscript. FF and QY assisted in phenotype analysis. HW and PZ analyzed and discussed the results. All authors agreed to publish the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.850062/full#supplementary-material

Supplementary Figure 1 | Gene transformation process of Physcomitrella patens and Arabidopsis. (A) Undigested P. patens protonema. (B, C) Digested P. patens protoplasts. (D, E) Screening of transgenic Pn2-ODD1 P. patens and Arabidopsis by antibiotic. (F, G) Identification of Pn2-ODD1 in P. patens and Arabidopsis by PCR analysis.

Supplementary Table 1 | All primers used for vector construction and gene expression analysis.

Supplementary Table 2 | Differential metabolites (36 up-regulate and 12 down-regulate) identified by the flavonoids metabolomics in 5-day-old WT and transgenic Arabidopsis.

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