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Authors' contributions

YY contributed to the design of the study and funding acquisition. FL contributed in drafting the manuscript and data analysis. TS, XT, MT and JG performed the experiments and obtained the data. All the author read and approved the final version of the manuscript.

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Bacillus amyloliquefaciens PDR1 from root of karst adaptive plant enhances *Arabidopsis thaliana* resistance to alkaline stress through modulation of plasma membrane H^+-ATPase activity

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Abstract

Exploration of native microbes is a feasible way to develop microbial agents for ecological restoration. This study was aimed to explore the impact of *Bacillus amyloliquefaciens* PDR1 from karst adaptive plant on the activity of root plasma membrane H^+-ATPase in *Arabidopsis thaliana*. *A. thaliana* was cultured in presence or absence of *B. amyloliquefaciens* PDR1 and its effects on the growth were evaluated by measuring the taproot length and dry weight. The rhizosphere acidification capacity was detected by a pH indicator, a pH meter and non-invasive micro-test techniques (NMT). The nutrient uptake was performed using appropriate methods. A combination of transcriptome sequencing and real-time quantitative polymerase chain reaction (qRT-PCR) was used to measure the expression of functional genes that regulate the plasma membrane H^+-ATPase activity in *A. thaliana* roots. Functional analysis was performed to understand how *B. amyloliquefaciens* regulates biological processes and metabolic pathways to strengthen *A. thaliana* resistance to alkaline stress. Here, we show that volatile organic compounds (VOCs) from *B. amyloliquefaciens* PDR1 promoted the growth and development of *A. thaliana*, enhanced the plasma membrane H^+-ATPase activity, and affected ion absorption in *Arabidopsis* roots. Moreover, *B. amyloliquefaciens* PDR1 VOCs did not affect the expression of the gene coding for plasma membrane H^+-ATPase, but affected the expression of genes regulating the activity of plasma membrane H^+-ATPase. Our findings illuminate the mechanism by which *B. amyloliquefaciens* regulates the growth and alkaline stress resistance of *A. thaliana*, and lay a foundation for wide and efficient application for agricultural production and ecological protection.

Keywords: *Bacillus amyloliquefaciens* PDR1; volatile organic compounds; H^+-ATPase activity; alkaline stress; *Arabidopsis thaliana*

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1. Introduction

Southwest China is the largest karst landform region with an area of more than 550,000 square kilometers worldwide. However, some karst forest ecosystems are severely degraded and entering a vicious circle of forest degradation, soil erosion, rocky desertification, and the environment is no longer suitable for vegetation growth. This poses a serious threat to the sustainable development of society. Therefore, research on methods for restoration and reconstruction of karst forest ecosystem has increasingly attracted national attention.

The basic rocks of karst geology are mainly carbonate rocks such as limestone and dolomite, which are covered with neutral to slightly alkaline calcareous soil rich in calcium and magnesium ions formed by the weathering of carbonate rocks. There are four most obvious characteristics of karst soil. First, the high content of calcium ions, up to l % - 3%, the average calcium content is several times that of non-karst soil. Second, the soil and water conservation ability are extremely poor, the surface is extremely dry and the water shortage is serious. Third, the soil pH is high, especially in rocky desertification areas. Fourth, with vegetation degeneration, the amount of organic acids secreted by the plant into the soil decrease, which increases soil pH to subsequently induce alkaline stress on plants. Therefore, plants growing in karst environment must have unique physiological and ecological adaptation mechanisms.

High soil pH (alkaline stress) is common for karst rocky desertification landscape, posing considerable threat to the ecological environment and agricultural production. Recent studies have shown that alkaline stress causes unique serious damage to plant growth and development compared to salt stress [1,2]. Compared with neutral salt stress, alkaline stress can increase the content of free calcium, magnesium and organic acid in the plant cells, but decrease the phosphate content [3,4].

Studies focused on the molecular mechanisms underlying plant response to alkaline stress are relatively rare. Transcriptome analysis of three alkali-tolerant plants such as wild soybean (*Glycine soja*) [5], *Tamarixhispida* [6] and bread wheat cultivar SR4 [7] revealed that alkaline stress can regulate the expression of a number of genes, which play an important role in the biological processes such as transcriptional regulation, nutrient absorption, signal transduction and oxidation reduction. The

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alkali-tolerant wild soybean alkaline stress response genes *GsPPCK3* (photosynthetic regulatory gene) [8] and *GsSKP21* (protein ubiquitination degradation-related gene) [9] overexpression plants show enhanced alkaline resistance.

The plasma membrane H^+-ATPase regulatory pathway is important for alkaline resistance in *Arabidopsis thaliana*. The plasma membrane H^+-ATPase is a family of proton pumps driven by ATP hydrolysis, which produces a transmembrane proton electrochemical gradient that is a prerequisite for plant cell nutrient uptake and growth [10]. Therefore, the activity of plasma membrane H^+-ATPase is critical for plants to adapt to alkaline stress (high pH) environments. The *Arabidopsis* plasma membrane H^+-ATPase isoform 2 (AHA2) mutant (*aha2*) has a reduced rhizosphere acidification capacity and is more sensitive to alkaline stress [11]. Studies have shown that protein post-translational modification is the main pathway to regulate the plasma membrane H^+-ATPase activity. Activation of the plasma membrane H^+-ATPase protein requires the penultimate threonine (Thr, amino acid 947 in AHA2) to be phosphorylated and bind to the 14-3-3 protein [12]. The 14-3-3 protein binds to the plasma membrane H^+-ATPase, keeping it in an active state. The SOS2-like protein kinase 5 (PKS5) inhibits the activity of AHA2 by interacting with 14-3-3 protein [13]. The *A. thaliana pks5* mutant secretes more protons in the rhizosphere and exhibits stronger alkaline resistance. Molecular chaperone J3 activates plasma membrane H^+-ATPase activity by inhibiting PKS5 [14]. Another important component of this signaling pathway is the auxin transporter PIN-FORMED2 (PIN2). In the *pks5*/*pin2* double mutant, the alkaline resistance of the *pks5* mutant is no longer stronger than that of the wild type [15]. The plasma membrane H^+-ATPase activity and proton efflux in *A. thaliana* roots also require auxin transporter gene *AUX1* [16]. In addition to auxin, abscisic acid (ABA) [17] also affects the activity of H^+-ATPase in roots, which in turn affects plants resistance to alkaline stress. Exogenous application of ABA reduces the activity of the root plasma membrane H^+-ATPase by 50% and this effect is lost in the mutant plants in which 6 ABA receptors are knocked out (*pyr1*/*pyl1*/*pyl2*/*pyl4*/*pyl5*/*pyl8*) or in plants overexpressing the HAB1 (ABA-inhibited protein phosphatase 2C isoform, ABA-inhibited protein phosphatase) [18].

In 2003, Ryu [19] first reported two strains of *Bacillus* spp. that promoted growth without direct contact with *A. thaliana*. This opened up a new field of research: the physiological and molecular mechanisms of bacterial volatile organic compounds (VOCs) affecting plant growth and stress

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resistance, and their agricultural and ecological applications. Previous studies showed that the VOCs of bacteria can affect the growth, salt tolerance, drought tolerance, disease resistance, insect resistance and other physiological processes of *A. thaliana*, wheat, tomato, pepper, cucumber and other plants [19-23]. *Bacillus subtilis* GB03 strain (originally classified as *B. subtilis*, classified as *B. amyloliquefaciens* [24] after genome sequencing) can promote the growth, and improve the ability of salt tolerance, and drought, insect and disease resistance of *A. thaliana* seedlings without direct physical contact. Previous studies found that *B. subtilis* GB03 significantly promotes the growth of *A. thaliana* [19]. It was also reported that *A. thaliana* seedlings exposed to VOCs produced by *B. subtilis* GB03 exhibit stronger resistance to the bacterial pathogen *Erwinia carotovora* subsp. Carotovora compared to *A. thaliana* exposed to *Escherichia coli* DH5α (as a control) [25]. Coculture with *B. subtilis* GB03 in a petri dish significantly increased the salt and drought tolerance of *A. thaliana* [26,27]. Plants exposed to *B. subtilis* GB03 exhibit greater protection against the generalist herbivore, *Spodopteraexigua* (beet armyworm, BAW) [28]. In addition to beneficial effects, a recent study showed that pathogenic fungi (*Stemphylium loti*) caused harmful effect on plants by producing the phytotoxin (tenuazonic acid) that specifically suppresses the activity of plasma membrane H^+-ATPase [29].

Studies have shown that VOCs from *B. subtilis* GB03 can regulate the gene expression in multiple functional classifications of *A. thaliana*, including flavonoid synthesis, auxin, ethylene, ABA and cytokinin synthesis and signaling, cell wall regulation, stress-related (such as heat shock protein), and signaling (such as transcription factors, kinases and phosphorylase) [30]. Furthermore, genetic and physiological analysis confirmed that VOCs from *B. subtilis* GB03 specifically regulate key functional genes and promote the absorption of iron [31] and sulfur [28] by *A. thaliana*, and affect the ABA [32], ethylene [25], cell division [30], auxin [30], salicylic acid and jasmonic acid [33] signaling pathways, as well as cell wall expansion pathway [30] in *A. thaliana*.

In this study, our main purpose was to explore the impact of *B. amyloliquefaciens* PDR1 isolated from karst adaptive plant on the activity of root plasma membrane H^+-ATPase in *Arabidopsis.* It was found that VOCs from *B. amyloliquefaciens* PDR1 promoted plasma membrane H^+-ATPase activity via protein post-translational modification. We stipulated that *B. amyloliquefaciens* PDR1 VOCs can regulate the expression of key genes regulating the activity of the plasma membrane H^+-ATPase in *A. thaliana* roots, alter the phosphorylation level of the enzyme, increase its activity, thereby ultimately

enhance the alkaline stress resistance of *A. thaliana*.

2. Material and Methods

2.1 Strain culture and seedling treatment

The strain used in this study, *B. amyloliquefaciens* strain PDR1, was isolated from root of karst adaptive plant *Cochlearia henryi*. *E. coli* DH5α has been proven to be a nongrowth-promoting strain of *A. thaliana* [19]. Here, it was used as a negative control to test the anti-alkali effect of VOCs from *B. amyloliquefaciens* PDR1 on *A. thaliana*, so as to ensure that the promoted growth and increased resistance to alkali stress were due to *B. amyloliquefaciens* PDR1. Bacteria strains were cultured in Luria broth (LB) solid medium in a 28°C incubator for 24 h in the dark. Then, a single colony was picked into the centrifuge tube containing 3 ml of LB liquid medium, and incubated in a shaker at 30°C for 12 h. For short-term storage before use, the obtained bacterial solution was placed in a refrigerator at 4°C. *A. thaliana* (Columbia ecotype) seeds were treated with 70% ethanol for 30 s and then surface-sterilized with 0.7% sodium hypochlorite for 15-20 min, then washed with distilled water for 4 times. The seeds were then sown on (0.6%) Murashige-Skoog (MS) medium and stored in 4°C refrigerator for 5 days. It was then placed in a greenhouse and grown for 3 days at 23°C under 16 h light/8 h dark conditions.

The *A. thaliana* seedlings and ddH_2O (water control), *E. coli* DH5α (bacteria control) or *B. amyloliquefaciens* PDR1 were placed on either side of a special culture dish (I-shaped plate, diameter 90 mm) containing an intermediate partition on 1/2 MS solid medium with agar content of 0.8%. This seedling and strain culture method ensures that the effect on plant growth is only due to the VOCs produced by the strain. KOH mother liquor was added to the 1/2 MS medium on which the *A. thaliana* side was placed to a final pH of 8.0 as alkaline stress treatment. Alkaline stress was not carried out on the strain side, and only 1/2 MS medium was used during the whole experiment. After 48 h treatment, the roots of *A. thaliana* were collected for subsequent analysis, including various physiological indicators and real-time quantitative polymerase chain reaction (qRT-PCR) analysis. Various physiological indicators were measured immediately after sample collection. *A. thaliana* root samples for qRT-PCR analysis were rapidly frozen in liquid nitrogen after collection and stored at -80°C. Since the water control and *E. coli* DH5α treatment have no significant difference in the development of

Arabidopsis roots (as described below), which is also consistent with previous researches [34,35], so the roots of *A. thaliana* collected form water control group were only used for morphological index (taproot length and dry weight) measurement. In the downstream experiments, only *E. coli* DH5α was used as the control (a bacteria control is more reasonable) to explore the role of VOCs from *B. amyloliquefaciens* PDR1 in promoting *Arabidopsis* resistance to alkali stress.

2.2 Determination of physiological indicators

The root length of *A. thaliana* was measured using Image J software (National Institutes of Health, USA). The stem and leaf parts of the *A. thaliana* seedlings were collected and simmered for 2 h at 105°C, followed by drying for 2 days at 60°C to constant weight. The root ion content was determined according to the method of Yang [4].

2.3 Determination of rhizosphere acidification capacity

Adding a pH indicator to the medium visually displays the change of medium pH. A 0.7 % agar medium containing the pH indicator bromocresol purple (0.006%, mass to volume ratio) was prepared. The pH of the medium was adjusted to 6.5 with KOH, and the medium was purple. When the pH of the medium drops to 5.2 or below, the color will turn yellow. *A. thaliana* seedlings were transferred to the bromocresol purple containing-medium, grown in greenhouse for 24 h, and photographed. *A. thaliana* seedlings of each treatment were immersed in distilled water (4 ml), and the pH of the distilled water was recorded after 2 h. Distilled water pH measurements were repeated 6 times using a pH meter (Fisher Scientific Accumet AP115, USA). In addition to pH indicator and pH meter, non-invasive micro-test techniques ~~(NMT; MA 01002, Younger USA LLC, Amherst, MA, USA; Xuyue Sci. & Tech. Co., Ltd., Beijing, China)~~ was used to measure the net flux of H^+ in *Arabidopsis* roots under four different treatments [36-39]. Briefly, the roots of *Arabidopsis* seedlings were fixed to the bottom of the petri dish using filter paper strips and resin blocks, exposing the root tips. The measuring solution (0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5 mM NaCl, and 0.3 mM MES, pH 5.8) was added to the petri dish until the roots were submerged. Then, the H^+ flow rate sensor was placed at a distance of about 10 μm from the root surface with the aid of a microscope. H^+ flux data was read via imFluxes V2.0 software (~~MA 01002, Younger USA LLC, Amherst, MA, USA~~) directly. The detection time lasted for 5 minutes, and with 15 biological replicates for each group.

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2.4 Measurement of root plasma membrane H^+-ATPase activity

The plasma membrane microcapsules were prepared according to the method of Shen et al. [40] and stored at -80°C. The activity of plasma membrane H^+-ATPase in *A. thaliana* roots was determined in 0.5 ml of a reaction solution containing 30 mM BTP/ MES/ 5 mM $MgSO_4$/ 50 mM KCl/ 4 mM TRIS-ATP at pH 6.5 as described previously [40]. The uniform size plasma membrane vesicles were obtained using Brij 58 (0.02% w/v). The reaction was initiated by adding 3-5 μg of membrane protein to the reaction solution, carried out at 30°C for 30 min and terminated with 1 ml of stop solution (2% (v/v) concentrated H_2SO_4, 5% (w/v) sodium lauryl sulfate, 0.7% (w/v) sodium molybdate). Then, 50 μl of 10% (w/v) ascorbic acid was added, and the color reaction of the phosphomolybdate complex was carried out for 30 min. The absorbance at 700 nm was measured with a spectrophotometer (BRS, Brussel, Belgium). The difference in ATPase activity between the reaction system and the inclusion of 0.1 mM sodium orthovanadate (inhibitor of plasma membrane H^+-ATPase) was calculated as the plasma membrane H^+-ATPase activity value. In each assay, a standard curve of phosphorus in the reaction mixture was used.

2.5 Total RNA extraction, RNA-seq library construction, and Illumina sequencing

For RNA-sequencing, total RNA was extracted from roots using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Purified mRNA was used for cDNA libraries preparation with the Illumina TruSeqTM RNA Sample Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's recommendations. Strand-specific sequencing was performed on the Illumina HiSeq 4000 system with paired-end 150-bp reads.

2.6 Differential gene expression and functional enrichment analysis

Raw RNA-seq reads were filtered to remove residual adaptor sequences and low-quality reads (quality score, Q > 20). FastQC was used to evaluate the quality of sequences before and after trimming with default seetings [41]. The resulting clean reads were aligned with Bowite2 and mapped to the *A. thaliana* reference genome using the spliced read aligner TopHat [42]. Transcripts were compared with the protein sequences in the Swiss-Prot database by using the basic local alignment search tool with a translated nucleotide query (BlastX) to eliminate protein-coding transcripts. The expression levels were standardized by the FPKM value using Cufflinks software [43]. Differential

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expression analysis was carried out using edgeR [44]. Genes were identified to be differentially expressed if they satisfied both false discovery rate (FDR) < 0.05 and $|Log_2$ (Fold Change)| > 0.585. To better understand the metabolic pathways of the identified genes, Gene Ontology (GO) pathway enrichment tests (Blast2GO software [45]) were performed to obtain related functional information of differentially expressed genes (DEGs).

2.7 Quantitative real-time PCR (qRT-PCR)

10-days old *A. thaliana* seedlings, which come from independent treated batches of plant, were collected from *E. coli* DH5α and *B. amyloliquefaciens* PDR1 treatment groups. Total root RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used as a template for qRT-PCR assay. cDNA was synthesized from 2 μg of DNase-treated total RNA based on M-MLV reverse transcriptase (TaKaRa, Tokyo, Japan) according to the manufacturer's protocol. qRT-PCR assays were conducted on a Step One™ Real-time PCR System (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ) using SYBR PrimeScript® RT-PCR Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. Each 10 μl qRT-PCR contained 5 μl of LBRBR, 5 μM of each primer and 1 μl of template. PCR reaction conditions comprised one cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 20 s. GAPDH was used as an internal reference. All relevant primer sequences were commercially synthesized and listed in **Table 1**. AHA gene-specific primers [46] were used to analyze the expression of the major root plasma membrane H^+-ATPase genes AHA1, AHA2, and AHA7 of *A. thaliana* roots. Relative expression levels were calculated using the 2-ΔΔCt formula. Three technical replicates were included.

2.8 Western blot analysis

To detect the expression level of plasma membrane H^+-ATPase and its phosphorylation level of penultimate Thr in response to *B. amyloliquefaciens* PDR1, polyclonal antibodies against the AHA2 (anti-H^+-ATPase) and the phosphorylated Thr947 of AHA2 (anti-$pThr_{947}$) were used for western blotting. The root tips (about 5mm) of Arabidopsis seedlings were cut and ground immediately in a mortar under ice bath conditions and then suspended in 100 μl SDS sample buffer (60 mM Tris-Hcl pH 6.8, 0.7 M 2-mercaptoethanol, 2% SDS, 4% glycerol, 0.01% bromphenol blue, 2 mM phenylmethylsulfonyl fluoride). The extracted proteins were separated by sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into nitrocellulose (NC) membrane (Bio-Rad Laboratories). Then, the membranes were probed with anti-H^+-ATPase and anti-$pThr_{947}$ as the primary antibodies overnight at 4°C. Next day, the membranes were reacted with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad Laboratories) as the secondary antibody, and electrochemiluminescence (ECL) reagent (Millipore, Billerica, MA, USA) was used to detect the signals.

2.9 Statistical Analysis

Data analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA.). The data were expressed as mean ± standard deviation (mean ± SD), and one-way ANOVA was used to analyze the significance of the differences between different groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 *B. amyloliquefaciens* PDR1 VOCs increase the resistance of *A. thaliana* to alkaline stress

Our experiments showed that *B. amyloliquefaciens* PDR1 VOCs significantly promoted the growth of *A. thaliana* compared to water control and *E. coli* treatment (**Fig. 1A**). In addition, we found that alkaline stress significantly reduced the taproot length and shoot dry weight of *A. thaliana*, and *B. amyloliquefaciens* PDR1 treatment alleviated the inhibition of alkaline stress. Compared to the none-stressed growth condition, the taproot length of *A. thaliana* exposed to ddH_2O, *E. coli* DH5α and *B. amyloliquefaciens* PDR1 under alkaline stress decreased by 57.84%, 63.64% and 55.65%, respectively (**Fig. 1B**). It is found that there is no significant difference in taproot length between the water control and *E. coli* DH5α treatment. However, the taproot length of *A. thaliana* exposed to *B. amyloliquefaciens* PDR1 was longer than that of water control and *E. coli* DH5α treatment, indicating that alkaline stress inhibited taproot development, but VOCs from *B. amyloliquefaciens* PDR1 significantly prompted the elongation of *A. thaliana* taproot under both normal and high pH conditions (**Fig. 1B**). A similar phenomenon was observed for the shoot dry weight of *A. thaliana* plants (**Fig. 1C**). These results demonstrated that VOCs from *B. amyloliquefaciens* PDR1 not only promote *A. thaliana* growth, but also increase *A. thaliana* resistance to alkaline stress.

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The high pH caused by alkaline stress directly affects the absorption of mineral elements by plants. We analyzed the ion content of the roots of *A. thaliana* under four treatment conditions. The results showed that alkaline stress increased the contents of Ca^{2+}, Mg^{2+} and total Fe ions in *A. thaliana* roots (**Fig. 2A-C**), while significantly reduced the contents of NH_4^+, NO_3^- and $H_2PO_4^-$ (**Fig. 2D-F**). Furthermore, compared to the *E. coli* DH5α strain exposure, exposure to VOCs from *B. amyloliquefaciens* PDR1 significantly increased the contents of NH_4^+, NO_3^- and $H_2PO_4^-$ in *A. thaliana* roots (**Fig. 2D-F**). These results indicated that *B. amyloliquefaciens* PDR1 VOCs can affect nutrients uptake and maintain anion-cation balance of *A. thaliana* under high pH conditions.

3.2 *B. amyloliquefaciens* PDR1 VOCs can enhance the rhizosphere acidification capacity and plasma membrane H^+-ATPase activity of *A. thaliana*

VOCs from *B. amyloliquefaciens* PDR1 significantly increased the growth of *A. thaliana* roots under alkaline stress conditions. The acidic growth theory suggests that lower pH in the extracellular space is a prerequisite for cell growth. To investigate the effect of VOCs from *B. amyloliquefaciens* PDR1 on the rhizosphere acidification capacity of *A. thaliana*, we used a pH indicator, a pH meter and NMT in combination to measure the pH in the medium. The results showed that compared with *E. coli* DH5α VOCs, VOCs from *B. amyloliquefaciens* PDR1 significantly increased the rhizosphere acidification capacity of *A. thaliana* seedlings under control or alkaline stress treatment (**Fig. 3A**). After 48 h of alkaline stress, the acidification ability of *A. thaliana* seedlings exposed to VOCs from *B. amyloliquefaciens* PDR1 was further enhanced. The acidification ability of *E. coli* DH5α VOCs treated *A. thaliana* seedlings was weaker than the unstressed treatment after 48 h of alkaline stress (**Fig. 3A**). pH meter and NMT were used to further quantify the rhizosphere acidification capacity of *A. thaliana* seedlings. It was found that *B. amyloliquefaciens* PDR1 VOCs could reduce the pH of distilled water to below 6 after 2 h (**Fig. 3B**), and the H^+ efflux in the roots of *A. thaliana* seedlings treated with VOCs from *B. amyloliquefaciens* PDR1 was significantly higher than those of *E. coli* DH5α treatment in the absence and presence of alkaline stress (**Fig. 3C**). Furthermore, treatment with VOCs from *B. amyloliquefaciens* PDR1 significantly increased the plasma membrane H^+-ATPase activity in *A. thaliana* roots (**Fig. 3D**). After alkaline stress, this effect was further enhanced and formed a strong contrast with *E. coli* DH5α VOCs treatment (**Fig. 3D**).

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3.3 Differential expression and GO pathway enrichment analysis in *A. thaliana* roots under four treatments

Compared with the *E. coli* group, a total of 4910 genes were differentially expressed (2256 up- and 2654 downregulated) in the *A. thaliana* roots treated with PDR1 (**Additional File 1**). The DEGs were annotated and classified into 605 GO classes including 364 biological processes (BP), 107 cellular components (CC) and 134 molecular functions (MF; **Additional File 2**). Under the BP category, 'response to salt stress', 'response to abscisic acid', 'response to cadmium ion', 'hyperosmotic salinity response' and 'root hair elongation' were prominently represented. Within these CC categories, 'cytosol', 'Golgi apparatus', 'cytoplasm', 'vacuole' and 'proton transporting two−sector ATPase complex, proton transporting domain' were significantly enriched. 'protein binding', 'structural constituent of ribosome', 'copper ion binding', 'metal ion binding' and 'proton−transporting ATPase activity, rotational mechanism' was significantly enriched in MF category (**Fig. 4A-C**).

Additionally, in another set of comparisons，a total of 122 (42 up- and 80 downregulated) genes were differentially expressed in the *A. thaliana* roots treated with $NaHCO_3$+PDR1 compared with the $NaHCO_3$+*E. coli* group (**Additional File 1**). 133 terms were enriched and could be classified as BP (72 terms), CC (10 terms), and MF (51 terms) (**Additional File 3**). As shown in **Fig. 4D-F**, 'cellular glucan metabolic process', 'carbohydrate metabolic process', 'indoleacetic acid biosynthetic process', 'regulation of root development' and 'nitrate transport' were significantly enriched in BP. 'extracellular region', 'cell wall', 'apoplast', 'glyoxysome' and 'plant−type cell wall' were the represented categories in CC. For the MF category, the largest proportion of genes were clustered into 'hydrolase activity, acting on glycosyl bonds', 'xyloglucan:xyloglucosyl transferase activity', 'lipid binding', 'hydrolase activity, hydrolyzing O−glycosyl compounds' and 'peptidase inhibitor activity' (**Fig. 4D-F**).

3.4 *B. amyloliquefaciens* PDR1 VOCs affect the expression of plasma membrane H^+-ATPase activity-regulating genes in *A. thaliana* root

The transcriptome sequencing analysis of four *A. thaliana* roots was carried out and the results indicated that the expression of all AHA genes encoding the plasma membrane H^+-ATPase enzyme were stable under the four treatment conditions in *A. thaliana* roots (**Fig. 5**). Moreover, the expression of *A. thaliana* roots major plasma membrane H^+-ATPase genes AHA1, AHA2, and AHA7 was verified

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by qRT-PCR analysis. As shown in the **Fig. 6A-C**, there was no significant difference in the expression levels of these three genes.

However, alkaline stress and treatment with VOCs from *B. amyloliquefaciens* PDR1 affected the expression of genes regulating the activity of the plasma membrane H^+-ATPase. Alkaline stress treatment induced the expression of plasma membrane H^+-ATPase activity promoting gene chaperone J3, downregulated the expression of the plasma membrane H^+-ATPase activity inhibitor protein kinase PKS5 (**Fig. 5; Fig. 6D, E**). Furthermore, we found that treatment VOCs from *B. amyloliquefaciens* PDR1 caused a significant downregulation in the expression of protein kinase PKS5 and a significant upregulation in the expression of chaperone J3 genes when compared to *E. coli* DH5α VOCs treatment (**Fig. 5; Fig. 6D, E**).

In addition, qRT-PCR analysis showed that VOCs from *B. amyloliquefaciens* PDR1 also affected the expression of auxin transport or signal transduction genes. The auxin transporter genes associated with plasma membrane H^+-ATPase activity, AT5G57090 (PIN2) and AT2G38120 (AUX1), were down-regulated by alkaline stress and treatment with VOCs from *B. amyloliquefaciens* PDR1 (**Fig. 6F, G**). However, under alkaline stress and treatment VOCs produced by *B. amyloliquefaciens* PDR1, the expression levels of PIN2 and AUX1 in *A. thaliana* were higher than the alkaline stress alone group. The gene involved in the regulation of plasma membrane H^+-ATPase activity in the ABA signaling pathway, AT1G72770 (HAB1), was also down-regulated by VOCs produced by *B. amyloliquefaciens* PDR1 and alkaline stress (**Fig. 6H**).

3.5 *B. amyloliquefaciens* PDR1 VOCs affect the phosphorylation level of plasma membrane H^+-ATPase in *A. thaliana* root

Western blot analysis was used to further confirm H^+-ATPase exhibit unchanged expression level during the different treatments, and confirmed that the activity of H^+-ATPase was regulated via protein post-translational modification. As shown in **Fig. 7A, 7B**, the results of western blotting indicated that there was no significant difference in the expression level of plasma membrane H^+-ATPase under the four treatments. However, *B. amyloliquefaciens* PDR1 treatment significantly increased the phosphorylation level of Thr_{947} in the H^+-ATPase when compared with *E. coli* DH5α treatment under both normal and high pH conditions (**Fig. 7C**).

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4. Discussion

Alkaline stress is a common and serious threat to agriculture. With the increase of human activities, the vegetation is destroyed, resulting in the reduction of organic acids secreted by plants, which makes the soil alkalinity increase and inhibits plant growth, eventually forming a vicious cycle [47]. However, studies on plant alkaline stress resistance and practical strategies for alkaline stress management are limited. Orhan et al. [48] showed that bacterial (*Bacillus* strains OSU-142 and M3) application decreased the soil pH level from 6.7 to 5.6-6.0, which provided a better organic growing conditions for plants. Besides, two plant growth-promoting rhizobacteria (*Agrobacterium rubi* A18 and *B. subtilis* OSU 142) were proved to be able to enhance resistance to alkaline stress by improving leaf physiology, vegetative growth and nutrient uptake in grapevine rootstocks [49]. However, the mechanism the mechanism by which bacteria help plants resist to alkaline stress is not clear. This study found for the first time that VOCs of *B. amyloliquefaciens* can improve the ability of *A. thaliana* to resist alkaline stress. This physiological ability is likely to be associated with an increase in the activity of the plasma membrane H^+-ATPase. Several reports indicated that the activity of plasma membrane H^+-ATPase is related to the ability of *A. thaliana* to resist alkaline stress [50-52]. Plasma membrane H^+-ATPase can regulate the proton concentration gradient on both sides of the plasma membrane and promote the absorption of cations [53,54]. It was previously reported that *B. subtilis* GB03 increases the rhizosphere acidification capacity of *A. thaliana* and promotes the absorption of iron ions [31]. This study found that in addition to the total iron ion content, *B. amyloliquefaciens* PDR1 increased the content of Ca^{2+} and Mg^{2+} in *A. thaliana*. Moreover, VOCs from *B. amyloliquefaciens* PDR1 affect nutrients uptake such as $NH4^+$, $NO3^-$ and $H2PO4^-$ under high pH conditions. This may be the physiological mechanism by which *B. amyloliquefaciens* PDR1 improves the resistance of *A. thaliana* to alkaline stress.

Based on the GO enrichment analysis of differentially expressed genes in *A. thaliana* roots under four treatments, it was found that the iron ion transport (GO:0006826) and metal ion binding (GO:0046872) were enriched in the biological process and molecular function respectively, which confirms the promotion of *B. amyloliquefaciens* PDR1 on metal ion content mentioned above. In addition, the DEGs also enriched in the pathways of nitrate transport (GO:0015706), amino acid import (GO:0043090) and response to nitrogen compound (GO:1901698), indicating *B. amyloliquefaciens*

VOCs might help *A. thaliana* to absorb and assimilate more nutrients. Furthermore, root development relevant pathways such as root hair elongation (GO:0048767), regulation of root development (GO:2000280) and regulation of lateral root development (GO:2000023) were enriched. Therefore, it is speculated that *B. amyloliquefaciens* PDR1 play a key role in plant growth of *A. thaliana*. Although *B. amyloliquefaciens* PDR1 increased the activity of plasma membrane H^+-ATPase of *A. thaliana*, it did not significantly affect the expression of plasma membrane H^+-ATPase genes in the roots. This was consistent with previous studies on the plasma membrane H^+-ATPase enzyme in *A. thaliana*, that is, protein post-translational modification like protein phosphorylation is an important pathway for the regulation of plasma membrane H^+-ATPase activity [55,56]. Due to its important physiological functions, the activity of plasma membrane H^+-ATPase is finely regulated and various external stimuli do not cause changes in AHA gene expression. Both transcriptome sequencing and qRT-PCR analysis indicated that the plasma membrane H^+-ATPase activity-promoting gene, molecular chaperone J3, was upregulated by *B. amyloliquefaciens* PDR1 VOCs. Protein kinase PKS5, the plasma membrane H^+-ATPase activity inhibitory gene [15]. Yang et al. [57] found that Ca^{2+}-binding proteins such as 14-3-3 and SOS3/SCaBP8 proteins could decode the calcium signal of salt stress and inhibit PKS5 activity to maintain sodium ion homeostasis in *Arabidopsis*. In our study, it was also found that the VOCs from *B. amylolyquefaciens* PDR1 downregulated the expression level of PKS5 and upregulated the phosphorylation level of plasma membrane H+-ATPase. This may be a specific molecular pathway for protein post-translational modification that regulated the plasma membrane H^+-ATPase activity in *A. thaliana* roots by VOCs from *B. amyloliquefaciens*.

The *B. amyloliquefaciens* VOCs have various physiological effects on plants, including promotion of reproductive functions and increased salt, drought, disease, and insect stress resistance [58-61]. The root plasma membrane H^+-ATPase is responsible for the generation of electrochemical gradients across the plasma membrane proton, which is a prerequisite for nutrient absorption and growth of plant cells [55]. Therefore, the high activity of the plasma membrane H^+-ATPase is a key factor in plant growth and stress adaptation. This study found that VOCs from *B. amyloliquefaciens* PDR1 promoted *A. thaliana* growth and increased resistance to alkaline stress in this plant species. The ability to increase the activity of plasma membrane H^+-ATPase in *A. thaliana* roots may be the key reason for the physiological effects of VOCs produced by *B. amyloliquefaciens* PDR1 on various aspects of plants.

Our study presents some limitations. Although it is found that PKS5 transcripts decreased upon *B. amyloliquefaciens* PDR1 treatment and the different expression level of phosphorylated Thr_{947} showed that the H^+-ATPase actually is post-translationally modified in response to *B. amyloliquefaciens* PDR1 VOCs, the molecular mechanism involved in the impact on 14-3-3 protein binding has not been explained clearly. For this reason, we plan to further investigate the mechanism by mass-spectrometry directed towards the Ser931 containing peptide. Alternatively, changes in the binding of 14-3-3 protein can be detected by 14-3-3 overlay-assay as Ser931 blocks 14-3-3 binding.

In conclusion, this study isolated a strain of *B. amyloliquefaciens* PDR1 from the rocky desertification soil of karst ecosystem and found that its VOCs can improve the resistance of *A. thaliana* to alkaline stress. We hypothesized that PDR1 VOCs can regulate the expression of key regulatory genes of the plasma membrane H^+-ATPase activity in *A. thaliana* roots, alter the phosphorylation level of the enzymes, increase its activity, and ultimately enhance the alkaline stress resistance of *A. thaliana*. We believe that the regulatory effects of VOCs produced by *B. amyloliquefaciens* PDR1 on the plasma membrane H^+-ATPase activity in *A. thaliana* roots may also be a key factor in the growth of *A. thaliana*. This study initially revealed the understanding of the mechanism by which *B. amyloliquefaciens* PDR1 regulates the growth and stress resistance of *A. thaliana*, and lays a foundation for its wide and efficient application in agricultural production.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

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617 **Table legend**

618 **Table 1. The primer sequences for qRT-PCR.**

Primers	5'-3'
GAPDH-F	AGGTCGGTGTGAACGGATTTG
GAPDH-R	TGTAGACCATGTAGTTGAGGTCA
AHA1-F	GGGGAATTCATGTCAGGTCTCGAAG
AHA1-R	GGGGAATTCTACACAGTGTAGTGAT
AHA2-F	GGGGAATTCATGTCGAGTCT-CGAAG
AHA2-R	GGGGAATTCTACACAGTGTAGTGAC
AHA7-F	GGGGAATTCATGACGGACATAGAAG
AHA7-R	GGGGAATTCTCAGATTGTGTAGTTGT
PKS5-F	TCGTCGGGATTGGATTTGTC
PKS5-R	TCCATCTCAAACCCATACTC
J3-F	TTCTACCTAAGCCGTCAAC
J3-R	CGTCATCATCATAAGCCTC
PIN2-F	CCGTGGGGCTAAGCTTCTCATCT
PIN2-R	AGCTTTCCGTCGTCTCCTATCTCC
AUX1-F	CACCATGTCGGAAGGAGTAGAAGC
AUX1-R	TCAAAGACGGTGGTGTAAAGCG
HAB1-F	CAACAGCAATATATGTATCTACG
HAB1-R	CCTCCATGGATCCTCCAAAATCAGAGATTTCC

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Figure legends

Figure 1: VOCs produced by *B. amyloliquefaciens* PDR1 increases the resistance of *A. thaliana* to alkaline stress. (A) The representative picture of *A. thaliana* treated with ddH_2O, *E. coli* DH5α and *B. amyloliquefaciens* PDR1 under normal and high pH conditions after 48 h, showing that PDR1 VOCs can improve the resistance of *A. thaliana* to alkaline stress. PDR1 VOCs treatment alleviated the effects of alkaline stress on *A. thaliana* taproot length **(B)** and shoot dry weight **(C)**. The data are presented as means ± SD (n=15). *P<0.05, **P<0.01 vs. ddH_2O, *E. coli* or $NaHCO_3$+ddH_2O, $NaHCO_3$+*E. coli* groups; ##P < 0.01 vs. ddH_2O, *E. coli* or PDR1 groups.

Figure 2: VOCs produced by *B. amyloliquefaciens* PDR1 affected nutrients uptake and ion balance in *A. thaliana*. The anion and cation content of the *A. thaliana* seedlings roots were determined. **(A)** calcium ion (Ca^{2+}), **(B)** magnesium ion (Mg^{2+}), **(C)** total iron ion, **(D)** ammonium ion (NH_4^+), **(E)** nitrate ion (NO_3^-), **(F)** dihydrogen phosphate ion ($H_2PO_4^-$). The data are presented as means ± SD (n=12). *P<0.05, **P<0.01 vs. *E. coli* or $NaHCO_3$+PDR1 groups; ##P < 0.01 vs. *E. coli* or PDR1 groups.

Figure 3: VOCs produced by *B. amyloliquefaciens* PDR1 enhanced the rhizosphere acidification capacity and plasma membrane H^+-ATPase activity of *A. thaliana*. (A) The rhizosphere acidification capacity of four treated *A. thaliana* seedlings was demonstrated in a medium containing the pH indicator (bromocresol purple). **(B)** The pH of distilled water after 2 h were measured using pH meter. **(C)** The net flux of H^+ in Arabidopsis roots under four different treatments was detected by non-invasive micro-test techniques (NMT). **(D)** Plasma membrane H^+-ATPase activity in the roots of *A. thaliana* seedlings was determined in the absence and presence of alkaline stress. The data are presented as means ± SD (n=15). **P<0.01 vs. *E. coli* or $NaHCO_3$+PDR1 groups; ##P < 0.01 vs. *E. coli* or PDR1 groups.

Figure 4: GO annotations pathway analyses of differentially expressed genes. (A-C) GO annotations pathway enrichment analysis of differentially expressed mRNAs between PDR1 and *E. coli* groups. **(D-F)** GO annotations pathway enrichment analysis of differentially expressed mRNAs between $NaHCO_3$+PDR1 and $NaHCO_3$+*E. coli* groups. BP: biological processes; MF: molecular function; CC: cellular component.

Figure 5: Heatmap showing the expression pattern of the functional genes involved in plasma membrane H^+-ATPase activity and its regulatory genes in *A. thaliana* roots under four treatments Values of fragments per kilobase of exon per million fragments mapped (FPKM) are log2 transformed, with blue indicating lowest level and red indicating highest level.

Figure 6: *B. amyloliquefaciens* PDR1 VOCs affect the expression of plasma membrane H^+-ATPase activity-regulating genes in *A. thaliana* root. The expression of H^+-ATPase activity-related genes **(A)** AHA1, **(B)** AHA2, **(C)** AHA7, **(D)** PKS5, **(E)** J3, **(F)** PIN2, **(G)** AUX1, **(H)** HAB1 in the roots of *A. thaliana* seedlings were measured using qRT-PCR. The control treatment (unstressed/*E. coli* DH5α VOCs treatment) value was set to 1 for each gene. The expression of the AHA gene remained stable during the four treatments. The data are presented as means ± SD (n=3). **P<0.01 vs. *E. coli* or $NaHCO_3$+PDR1 groups; ##P < 0.01 vs. *E. coli* or PDR1 groups.

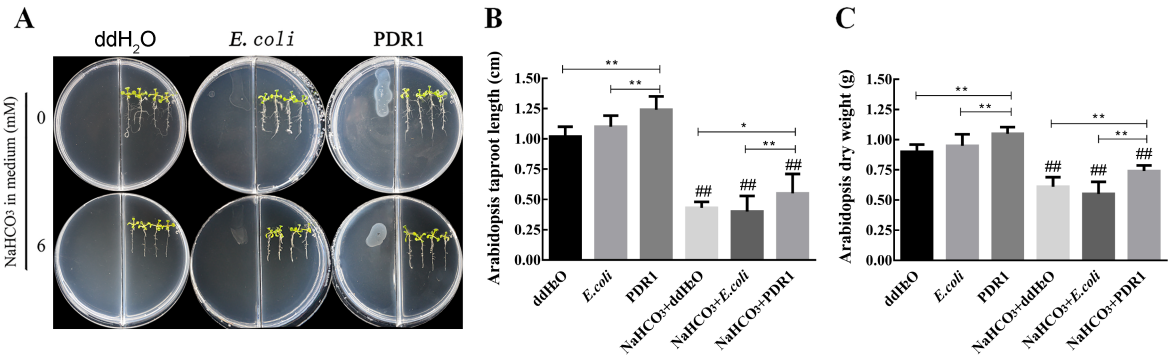
Figure 7: Expression level of plasma membrane H^+-ATPase and its phosphorylation level of penultimate Thr in response to *B. amyloliquefaciens* PDR1 were detected by western blotting. (A) Western blot revealed the levels of anti-H^+-ATPase and anti-$pThr_{947}$. β-actin was used as an internal reference. The H^+-ATPase **(B)** and H^+-ATPase phosphorylation **(C)** levels were determined using anti-$pThr_{947}$ and H^+-ATPase polyclonal antibodies. *P<0.05, **P<0.01 vs. *E. coli* or $NaHCO_3$+PDR1 groups; ##P < 0.01 vs. *E. coli* or PDR1 groups.

Additional Files

Additional File 1: The detailed information on the differentially expressed genes in *A. thaliana* roots under four treatments.

Additional File 2: GO annotations pathway enrichment analysis of differentially expressed genes in PDR1 and *E. coli* groups.

Additional File 3: GO annotations pathway enrichment analysis of differentially expressed genes in $NaHCO_3$+PDR1 and $NaHCO_3$+*E. coli* groups.



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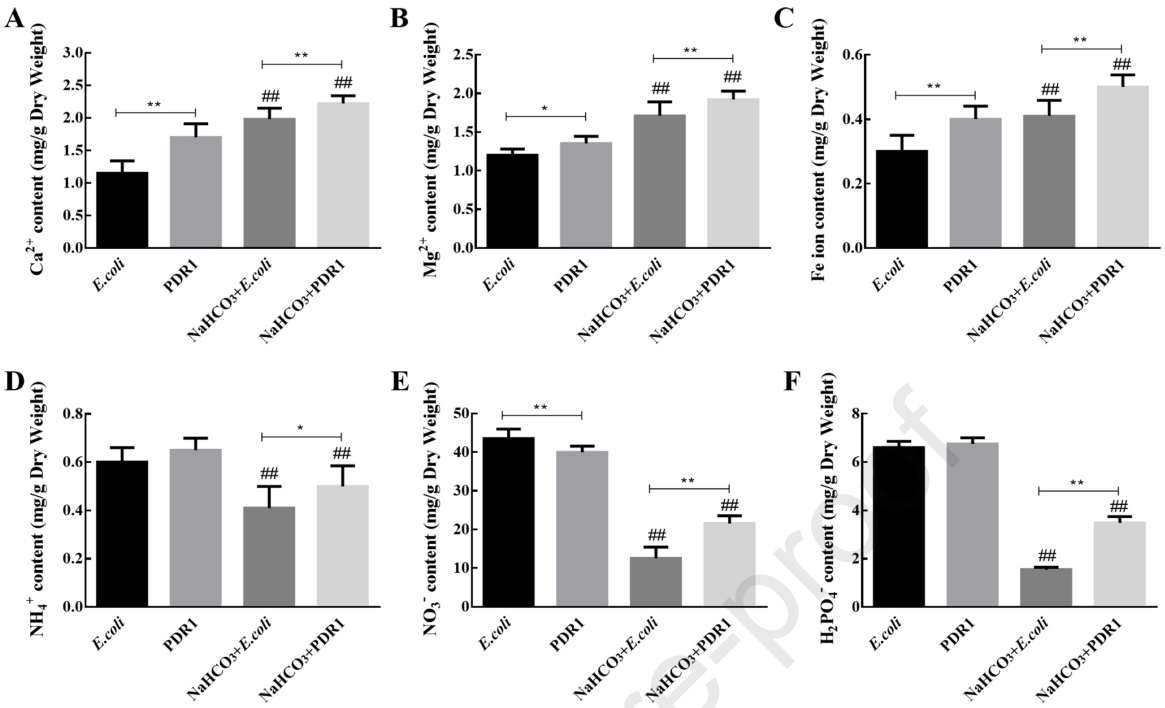

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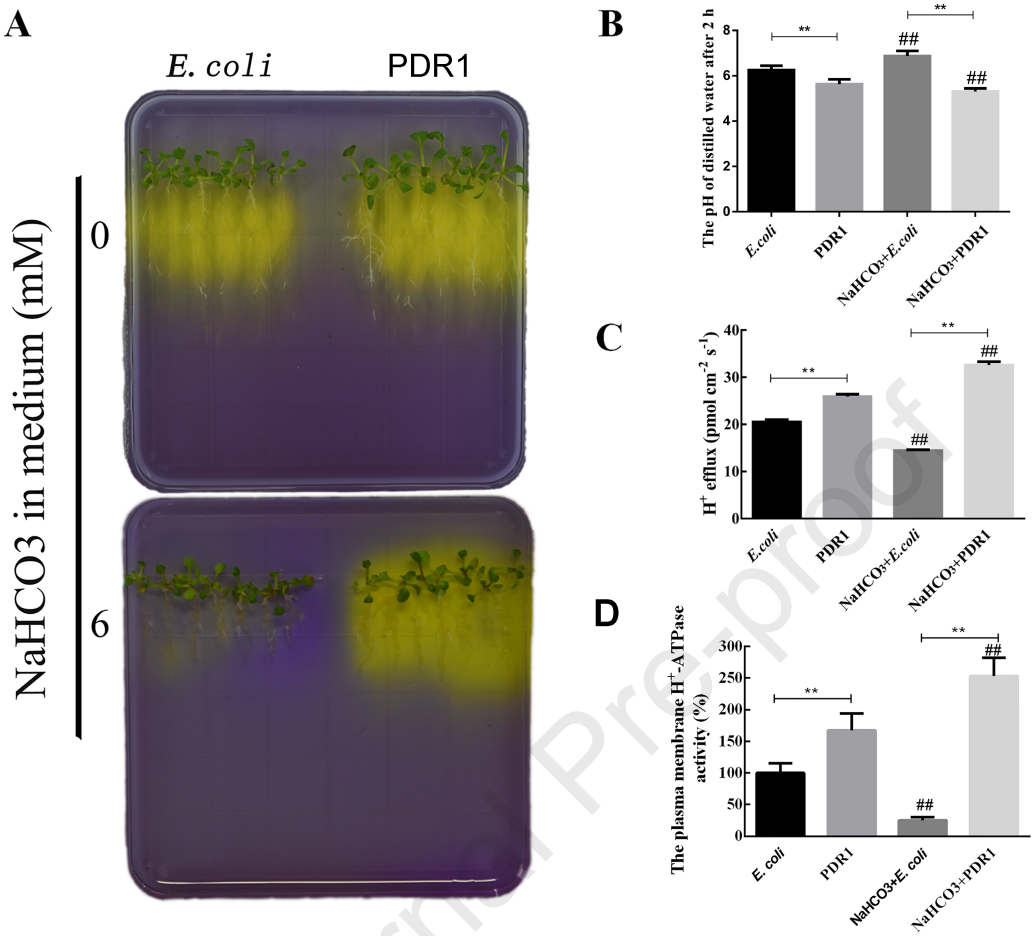

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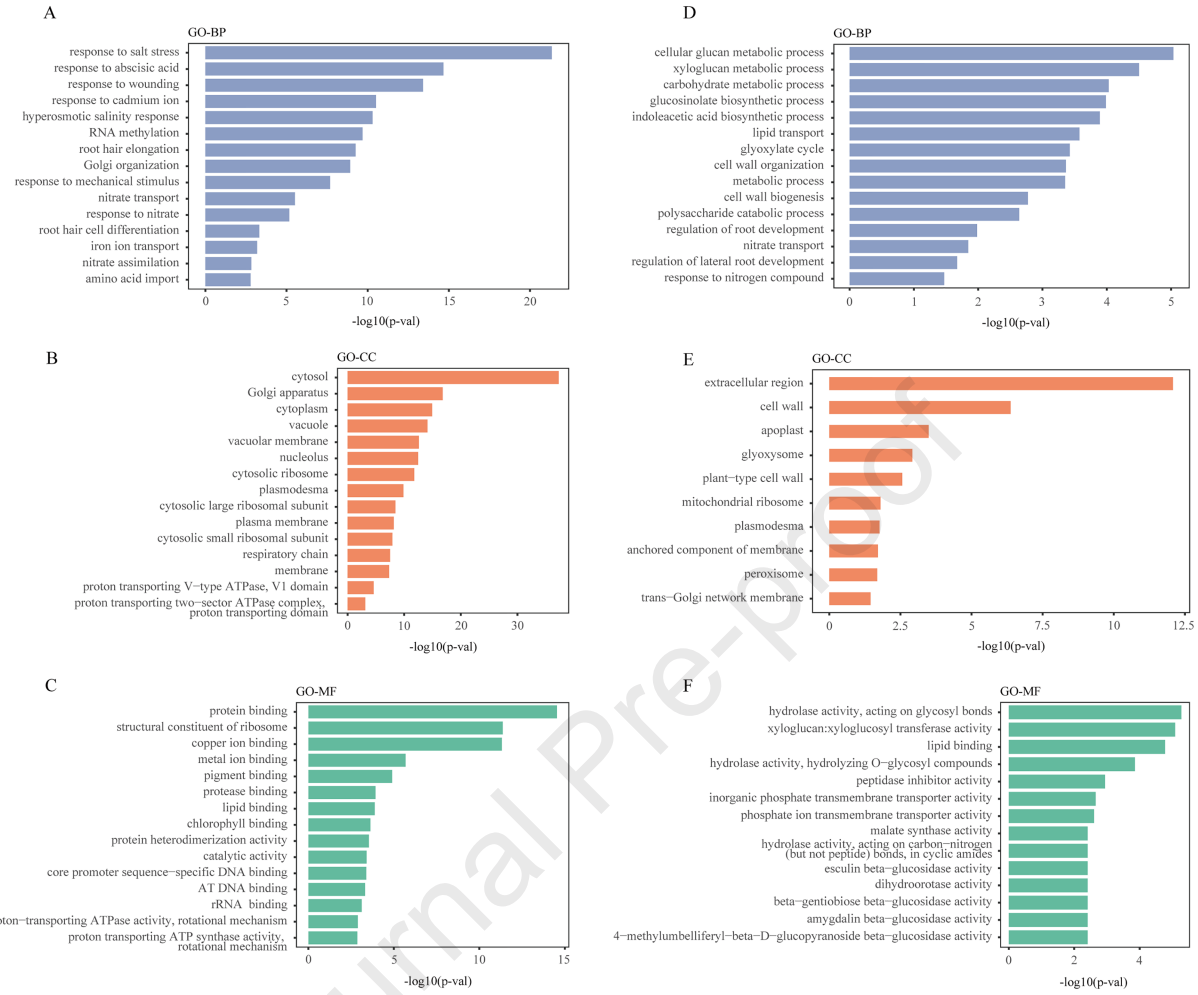
PDR1 vs *E. coli*

NaHCO3+PDR1 vs NaHCO3+*E. coli*



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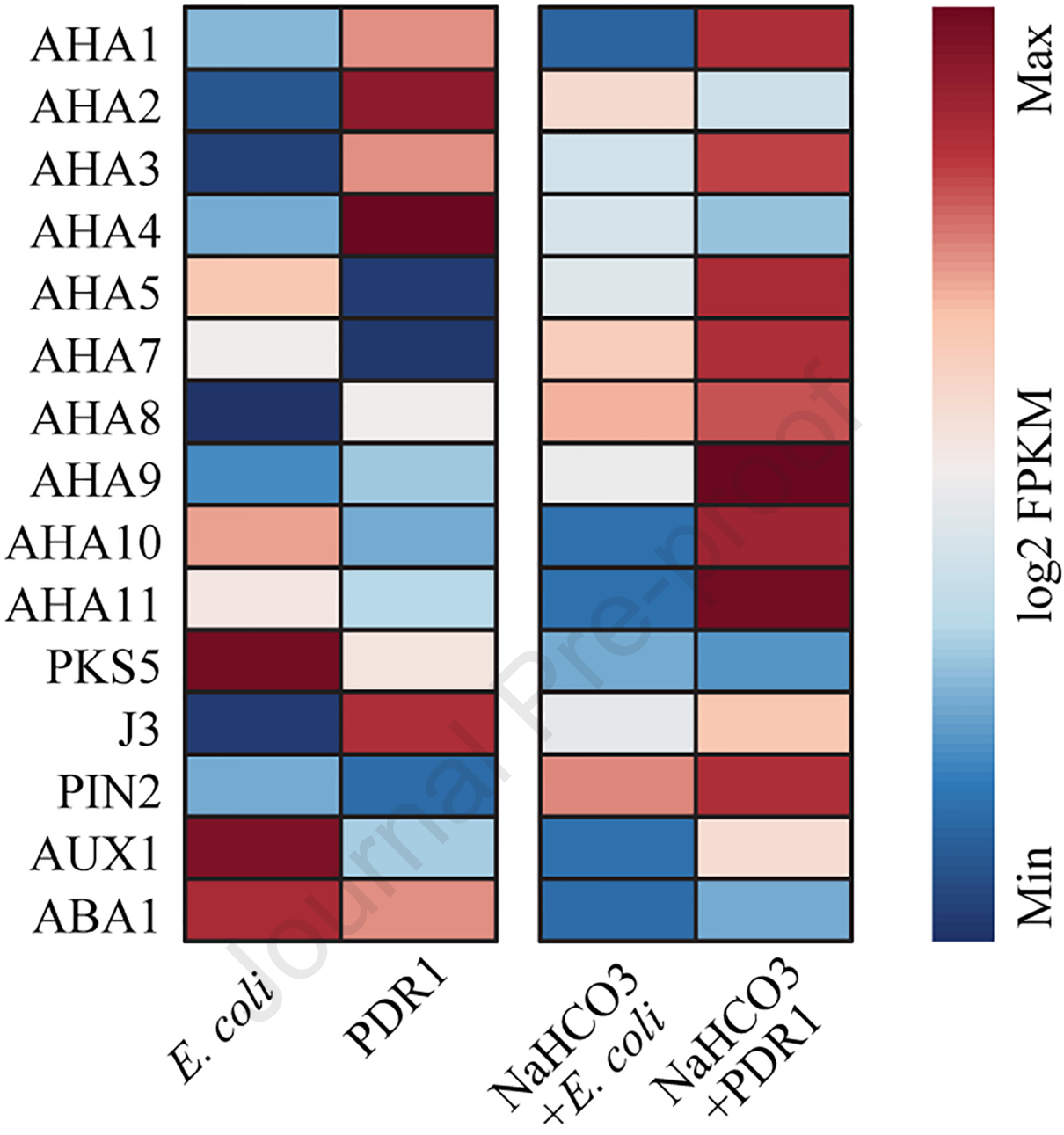

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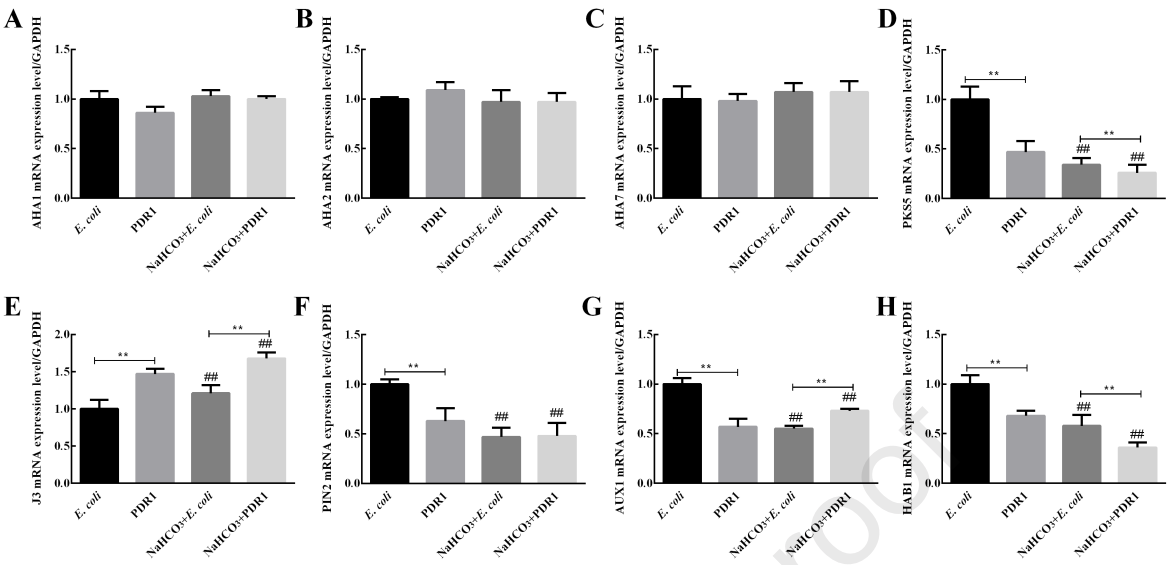

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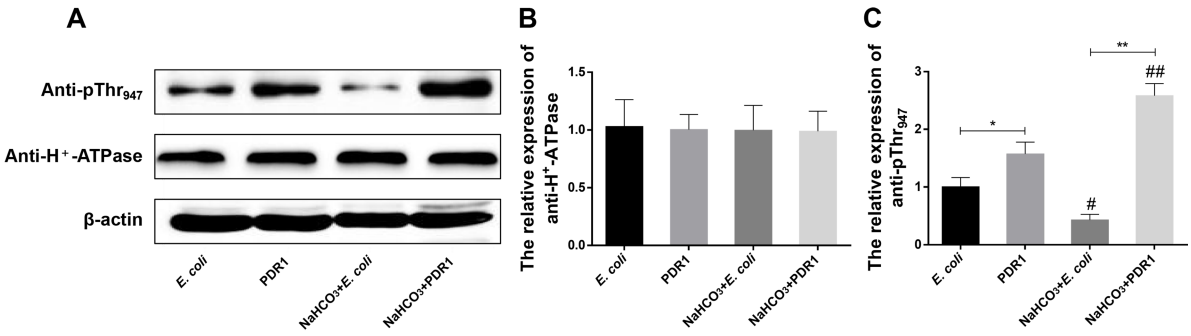

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Highlights

- Effect of VOCs on the activity of H^+-ATPase in *A. thaliana* has been explored.
- VOCs from *B. amyloliquefaciens* PDR1 prompted *A. thaliana* roots to absorb ions.
- VOCs from *B. amyloliquefaciens* PDR1 improved rhizosphere acidification capacity.
- VOCs from *B. amyloliquefaciens* PDR1 facilitated *A. thaliana* to resist acidic stress.

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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