Transcriptomic and phosphoproteomic profiling and metabolite analyses reveal the mechanism of NaHCO₃-induced organic acid secretion in grapevine roots

Guangqiang Xiang, Wanyun Ma, Shiwei Gao, Zhongxin Jin, Qianyu Yue and Yuxin Yao*

Abstract

Background: Organic acid secretion is a widespread physiological response of plants to alkalinity. However, the characteristics and underlying mechanism of the alkali-induced secretion of organic acids are poorly understood.

Results: Oxalate was the main organic acid synthesized and secreted in grapevine (a hybrid of Vitis amurensis, V. berlandieri and V. riparia) roots, while acetate synthesis and malate secretion were also promoted under NaHCO₃ stress. NaHCO₃ stress enhanced the H⁺ efflux rate of grapevine roots, which is related to the plasma membrane H⁺-ATPase activity. Transcriptomic profiling revealed that carbohydrate metabolism was the most significantly altered biological process under NaHCO₃ stress; a total of seven genes related to organic acid metabolism were significantly altered, including two phosphoenolpyruvate carboxylases and phosphoenolpyruvate carboxylase kinases. Additionally, the expression levels of five ATP-binding cassette transporters, particularly ATP-binding cassette B19, and two Al-activated malate transporter 2 s were substantially upregulated by NaHCO₃ stress. Phosphoproteomic profiling demonstrated that the altered phosphoproteins were primarily related to binding, catalytic activity and transporter activity in the context of their molecular functions. The phosphorylation levels of phosphoenolpyruvate carboxylase 3, two plasma membrane H⁺-ATPases 4 and ATP-binding cassette B19 and pleiotropic drug resistance 12 were significantly increased. Additionally, the inhibition of ethylene synthesis and perception completely blocked NaHCO₃-induced organic acid secretion, while the inhibition of indoleacetic acid synthesis reduced NaHCO₃-induced organic acid secretion.

Conclusions: Our results demonstrated that oxalate was the main organic acid produced under alkali stress and revealed the necessity of ethylene in mediating organic acid secretion. Additionally, we further identified several candidate genes and phosphoproteins responsible for organic acid metabolism and secretion.

Keywords: Grapevine, Transcriptome, Phosphoproteome, Organic acid secretion, Alkali stress

Background

Soil alkalinity is an important environmental problem, and alkali stress primarily caused by NaHCO₃ and Na₂CO₃ severely affects crop growth and development in more than 434 million hectares of land worldwide [1]. Compared with neutral salt stress, alkali stress not only causes osmotic stress and ion injury but also leads to high pH injury [2, 3]; therefore, alkali stress is more destructive than salt stress [4]. However, in contrast to the extensive studies on plant salt tolerance, much less attention has been focused on exploring the mechanisms underlying alkali stress tolerance.

Under alkali stress, plants must regulate intracellular pH and the pH outside roots to maintain root functions. Organic acids play a key role in regulating the cell and rhizosphere pH levels. It was reported that organic acid metabolism is closely correlated with alkali stress tolerance [4]. Puccinellia tenuiflora roots accumulate and secrete citric acid into the rhizosphere in response to alkali stress [5]. HCO₃⁻ induces the production of...
malate, succinate, and citrate in rice roots [6] and malate in maize roots [7]. Additionally, organic acids have been reported to function in plant adaptation to other abiotic stresses. For example, cotton and alfalfa accumulate more citric acid under drought and salt stresses, respectively [8, 9]. Plants accumulate organic acids in cells and secrete them into rhizospheres when facing Al, P and Cd toxicity [10–12]. With regard to the mechanisms of alkali-induced organic acid synthesis and secretion, some key enzymes involved in organic acid metabolism, such as citrate synthase, malate synthase and isocitrate lyase, have been suggested to determine alkali stress tolerance [13]. Plasma membrane H+-ATPases, such as Arabidopsis AHA2 and AHA7, are necessary for proton secretion for plant tolerance to alkali stress [11, 14]. Additionally, some studies have demonstrated that ethylene and auxin have roles in the regulation of root H+ secretion and alkali stress by regulating H+-ATPase [15, 16].

To date, the pathways of organic acid metabolism in the cell and extrusion out of the cell under alkali stress remain largely unknown in grapevine. Genome-scale analysis of gene expression profiles is a powerful method to reveal plant abiotic stress tolerance mechanisms [17, 18]. For example, transcriptome profiling reveals the genetic basis of alkalinity tolerance in wheat [18] and gene networks responsive to NaHCO3 stress in Tamarix hispida [17]. Quantitative and comprehensive insights into the mRNA transcriptome will contribute to unraveling the key mechanism of organic acid secretion in response to alkali treatment. On the other hand, phosphorylation represents one of the most important posttranslational modification functions in diverse biological pathways. The activities of several proteins related to organic acid synthesis and extrusion, such as phosphoenolpyruvate carboxylase (PEPC) and H+-ATPase [19, 20], have been shown to be regulated by phosphorylation. An analysis of the comprehensive phosphorylation modifications induced by alkali stress is needed to better understand the process of organic acid secretion under alkali stress.

Grapevines are an economically important fruit crop worldwide that grow as deep-rooted perennial plants, and their growth and fruit quality are largely influenced by soil alkalinity and salinity. The majority of commercial rootstocks and varieties used in viticulture are moderately sensitive to alkali stress. However, the candidate rootstock cultivar A15 possesses strong alkali tolerance and is a suitable material for identifying alkali tolerance-associated genes. Additionally, A15 was identified to have an higher capacity to secrete organic acids than other grapevine rootstocks with moderate or weak tolerance to alkali stress [21]. The present study determined the accumulation and secretion of organic acids and H+ efflux at different time points after NaHCO3 treatment. Thereafter, the key genes and phosphoproteins involved in the above processes were identified using transcriptomic and phosphoproteomic analyses. Additionally, the roles of ethylene and indoleacetic acid (IAA) in mediating organic acid secretion under alkali conditions were evaluated. The results provide valuable information for dissecting the metabolism and regulatory pathways of alkali stress tolerance in grapevine roots.

Results
Effects of NaHCO3 on the synthesis and secretion of organic acids in grapevine roots
A total of six organic acids were identified, oxalate, malate, tartrate, succinate, citrate and acetate. Oxalate was the main organic acid and accounted for 69.5–71.3% of the sum of the six organic acids in the control roots. In contrast, the five other organic acids, particularly citrate, showed relatively low concentrations (Fig. 1a-f). Similar differences in organic acid concentrations were also found in the culture solution (Fig. 1g–l); therefore, oxalate was the main organic acid synthesized and secreted by grapevine roots. The content of oxalate in the NaHCO3-treated roots was significantly enhanced from 3 to 24 h after treatment (HAT) and reached a maximum increase of 87.1% at 12 HAT compared to that in the control roots (Fig. 1a). The increased synthesis of oxalate promoted oxalate secretion and led to continuous accumulation in the culture solution; the oxalate content in the NaHCO3 culture solution reached 1.56 times that of the control solution at 24 HAT (Fig. 1g). Additionally, NaHCO3 treatment continuously significantly enhanced acetate synthesis but did not promote its secretion (Fig. 1f, l). In contrast, the other organic acids were generally not affected by NaHCO3 treatment. However, notably, the content of malate in the culture solution containing NaHCO3 was substantially increased at all time points (Fig. 1h). On the other hand, NaCl treatment as a positive control produced different effects on organic acid synthesis and secretion, e.g., NaCl imparted a reduced and stronger influence on oxalate and malate secretion, respectively, than NaHCO3 (Fig. 1g, h). Therefore, the changes in organic acid synthesis and secretion under NaHCO3 were attributed to both Na+ and HCO3−.
DAT (Fig. 2a). In contrast, the roots treated with NaHCO₃ began to produce a clear yellow color at 0.5 DAT and produced large areas of yellow color at 3 and 5 DAT (Fig. 2c), indicating that large amounts of acidic substances had accumulated. Additionally, the NaCl treatment as a positive control led to a clear yellow color, but the area and intensity were smaller than those under NaHCO₃ treatment (Fig. 2b, c); therefore, the production of acidic substances was attributed to both Na⁺ and \( \text{HCO}_3^- \). Moreover, the H⁺-ATPase inhibitor Na₃VO₄ almost completely inhibited the production of yellow color (Fig. 2d), suggesting that acid secretion is related to the PM H⁺-ATPase activity. Notably, the production of yellow color was accompanied by the occurrence of newly grown roots (Fig. 2a-c), and the production of yellow color and new roots was almost completely inhibited by the simultaneous treatment of NaHCO₃ plus Na₃VO₄ (Fig. 2d). Further experimentation using solid medium, half of which contained Na₃VO₄, indicated that root growth and H⁺ secretion were substantially affected by H⁺-ATPase activity (Fig. 2e). Therefore, NaHCO₃ treatment induced H⁺ secretion, which was accompanied by new root growth.

On the other hand, H⁺ flux was determined using noninvasive microtest technology to further determine H⁺ secretion under NaHCO₃ treatment (Fig. 3a). High negative values of H⁺ flux prior to treatment indicated the influx of H⁺ into root cells. In contrast, the H⁺ flux gradually changed to efflux, as indicated by the positive
values in the control and NaHCO3-treated roots from 1 HAT. Compared to the low H+ efflux rate of the control roots, the H+ efflux rate of the roots treated with NaHCO3 was very high at 6 and 12 HAT. Additionally, the highest H+ efflux rate in the treated roots occurred at 12 HAT. On the other hand, the NaHCO3-treated roots possessed higher H+-ATPase activity than the control roots, and the highest activity of the treated roots was found at 12 HAT, which was 0.46-fold higher than that in the control (Fig. 3b).

Identification of the changes in the transcriptome profile of grapevine roots in response to NaHCO3

To explore the mechanism underlying the alkali stress-induced oxalate secretion, RNA-Seq analysis of the control and NaHCO3-treated vine roots was conducted to quantify gene changes. A total of 3232 and 1714 genes were up- and downregulated by at least one-fold in the NaHCO3-treated vine roots, respectively (Fig. 2), demonstrating that NaHCO3 treatment caused massive transcriptional reprogramming in the vine roots. All of the annotated differentially expressed genes (DEGs) were associated with 20 biological processes. The process of carbohydrate metabolism contained the most DEGs; additionally, biosynthesis of other secondary metabolites, environmental adaptation, amino acid metabolism and signal transduction were also clearly changed biological processes (Fig. 4a).

In the carbohydrate metabolism process, the gene expression of seven genes involved in oxalate and malate metabolism was significantly altered by NaHCO3 treatment (Fig. 2 and Fig. 4b). Two PEPCs and their kinases...
(PEPCKs) were significantly upregulated, positively contributing to the biosynthesis of oxaloacetate and thereby providing the substrate for oxalate and malate biosynthesis. An oxalate--CoA ligase gene (OCL) and two NADP-malic enzyme (ME) genes were significantly downregulated, which decreased the degradation of oxalate and malate, respectively. Additionally, the expression of five ATP-binding cassette (ABC) transporters, belonging to the process of membrane transport, was substantially increased, and in particular, the expression of ATP-binding cassette B19 (ABCB19) increased 9.66-fold. Two aluminum-activated malate transporters (ALMTs) exhibited more than 5.0-fold higher expression in the NaHCO₃-treated roots than in the control roots. Plasma membrane (PM) H⁺-ATPases PMA2 and AHA11 were only detected in the NaHCO₃-treated roots but showed very low values of reads per kilobase of transcript per million mapped reads (RPKM), suggesting that they might not be the primary proton pump in grapevine roots.

With respect to the signal transduction process, the most significantly altered pathway was the ethylene signaling pathway, for which 51 genes related to ethylene biosynthesis and signaling were transcriptionally modified. Six 1-aminoacyclop propane-1-carboxylic acid (ACC) synthases (ACSSs) were substantially upregulated by NaHCO₃ treatment; in contrast, two ACC oxidases (ACOs) were transcriptionally changed, and ACO1 was downregulated 2.97-fold (Additional file 1: Table S1). Additionally, IAA metabolism and signaling were also significantly changed. The expression of four genes related to IAA metabolism and signaling was upregulated more than 5-fold, including indole-3-acetic acid-amido synthetase (GH3) and three IAA responsive factors (IAA12, SAUR32 and SAUR40). Moreover, six protein kinases located in the plasma membrane were substantially upregulated by NaHCO₃ treatment, suggesting that plasma membrane proteins were regulated by phosphorylation.

On the other hand, 34 differentially expressed genes, which contained genes involved in organic metabolism and transport and hormone biosynthesis and signaling, were detected by qRT-PCR at different times after NaHCO₃ treatment. Similar expression changes in the DEG tag profiles were found, which not only validated the reliability of the DEGs but also demonstrated their expression patterns under alkali stress (Fig. 4c).

**Quantitative analysis of phosphoproteins with phosphorylation levels significantly changed in response to NaHCO₃**

We identified 2669 unique phosphoproteins, collectively containing 6312 nonredundant phosphorylation sites. Among those phosphorylation sites, 5404 (85.6%) were found at serine, 877 (13.9%) were found at threonine
and 31 (0.49%) were found at tyrosine residues. Of the 2669 phosphoproteins, 2141 phosphoproteins were identified, which contained different quantities of phosphorylation sites (from 1 to 25); a total of 1822 and 608 phosphoproteins contained one and two phosphorylation sites, respectively (Additional file 2: Table S2).

When comparing the phosphorylation levels between the NaHCO₃-treated and control samples, a total of 197 phosphoproteins (270 phosphorylation sites) showed a significant change (ratio ≥ 1.5, P < 0.05), with 107 upregulated and 163 downregulated (Additional file 2: Table S2). The 197 phosphoproteins were annotated using Blast2GO according to the biological process, cellular component and molecular function (Fig. 5a). The phosphoproteins were classified into 11 biological processes, with metabolic process, cellular process and localization as the top three categories. For the molecular function, phosphoproteins were classified into 9 categories, and the top 3 categories were binding, catalytic activity and transporter activity. For the cellular components, cell part, cell and membrane possessed the highest number of phosphoproteins (Fig. 5a). The phosphoproteins modified by NaHCO₃ were primarily localized in the nucleus, cytoplasm, chloroplast and plasma (Fig. 5b). Additionally, Motif-X analysis identified 16 significantly enriched motifs (Fig. 5c).

Aconitate hydratase (ACH) and PEPC3–1, which are involved in oxalate and malate synthesis, were significantly phosphorylated in the NaHCO₃ treatment compared to the control (Fig. 4b; Table 1). The phosphorylation levels of two plasma membrane-localized ATPases (ATPase4–1 and particularly ATPase4–2) were substantially enhanced, which are responsible for H⁺ efflux and provide energy for the transport of oxalate and malate across the plasma...
membrane. Additionally, two plasma membrane ABC transporters ABCB19–2 and pleiotropic drug resistance 12 (PDR12), potential transporters of oxalate, were also significantly phosphorylated. Moreover, the phosphorylation levels of two plasma membrane- localized serine/threonine-protein kinases (STY46 isoform X1 and CDL1), ACO4 and ACO11 for ethylene synthesis and EIN2 for ethylene signaling were significantly changed (Fig. 4b; Table 1).

Organic acid secretion by vine roots involves ethylene and IAA

Transcriptomic and/or phosphoproteomic profiling indicated that the metabolism and signaling of IAA and ethylene were significantly altered by NaHCO₃ treatment. To reveal the role of ethylene and IAA in mediating NaHCO₃-induced organic acid secretion, the changes in ethylene and IAA under NaHCO₃ treatment and the effects of the inhibition of ethylene and IAA biosynthesis and/or signaling on organic acid secretion were determined. Compared with the control, NaHCO₃ treatment significantly increased ethylene production at 3 and 6 HAT but substantially decreased ethylene production at 12 and 24 DAT. In contrast, the IAA content was substantially reduced by NaHCO₃ at 6 DAT, and the decrease in the IAA content reached 57.6% at 24 HAT (Fig. 6a, b). Additionally, the inhibition of ethylene biosynthesis and perception via aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) completely blocked NaHCO₃-induced organic acid secretion, and the inhibition of IAA biosynthesis via 1-N-naphthylphthalamic acid

Fig. 5 The distribution of differentially phosphorylated proteins (a, b) and significantly enriched phosphorylated sites (c). The 197 phosphorylated proteins were classified according to their biological process, cellular component, molecular function and subcellular location (a, b). The Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (www. http://www.ebi.ac.uk/GOA/). Soft motif-x was used to analyze the model of sequences constituted with amino acids in specific positions of modify-21-mers (10 amino acids upstream and downstream of the site, but phosphorylation with modified-13-mers was 6 amino acids upstream and downstream of the site) in all protein sequences. All of the database protein sequences were used as background database parameters and other parameters with default

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(NPA) significantly reduced NaHCO$_3$-induced organic acid secretion (Fig. 6c, d). Therefore, ethylene signaling is necessary to regulate organic acid secretion under NaHCO$_3$, and IAA also participates in the process; additionally, their roles are largely affected by their concentrations in plants.

**Discussion**

Accumulation and secretion of oxalate are important physiological responses of grapevine roots to alkali stress. Plant roots are in direct contact with the soil and can adjust to an adverse rhizosphere environment by secreting a large amount of compounds, which is the
most direct and obvious response of roots to environmental stresses [5]. Under alkali stress, the accumulation of organic acids is observed in many plants. Oxalate was highly accumulated and secreted by grapevine roots under alkali stress (Fig. 1a, g). A large amount of oxalate was also found in the roots of Kochia sieversiana and Suaeda glauca under alkali stress [22, 23]. In contrast, Chloris virgata was found to predominantly accumulate citrate, and wheat roots secrete large amounts of lactic, acetic and formic acids under alkali stress [24, 25]. The accumulation and secretion of organic acids, including oxalate, citrate and malate, play important roles in osmoregulation, pH adjustment, and ionic balance maintenance by providing a negative charge under alkali and salt stresses [5, 22, 23]. This study demonstrated that both NaHCO₃ and NaCl stimulated the secretion of organic acids, but NaHCO₃ created a more acidic rhizosphere environment (Fig. 2b, c). Additionally, NaHCO₃ and NaCl imparted different effects on oxalate, acetate and malate (Fig. 1). Similarly, previous reports have also demonstrated that alkali and salt stress have different effects on organic acid metabolism and secretion. For example, the concentration of citrate, the most dominant organic acid, is substantially increased by alkali stress but decreased by salt stress in Puccinellia tenuiflora roots; additionally, alkali stress causes the secretion of citric acid into the rhizosphere, while salt stress does not [5]. Different changes in citrate and malate were also found in Chloris virgata under alkali and salt stresses [25].

Therefore, organic acid secretion is a widespread physiological response of plants, but oxalate is the primary organic acid synthesized and secreted by vine roots. Additionally, −HCO₃ plays a key role in inducing oxalate synthesis and secretion in grapevine roots.
The possible pathway of organic acid synthesis and secretion based on the key genes and phosphoproteins under alkali stress

Three pathways for oxalate biosynthesis have been proposed in plants, i.e., the glycolate/glyoxylate pathway, the ascorbate pathway and the oxaloacetate (OAA) pathway [26, 27]. Under alkali stress, substantial amounts of HCO3− enter the root and provide the substrate for PEPCs, which catalyze the β-carboxylation of PEP to OAA using HCO3 in the cytosol in an irreversible process [28]; thereafter, OAA can be converted to oxalate, acetate and malate (Fig. 4b). In Arabidopsis, the pepc3 mutant abolished the salt-stress-induced increase in malate, suggesting the role of AtPEPC3 in regulating organic acid synthesis [29]. The significant upregulation of the expression of two PEPC3 genes under alkali stress might enhance OAA synthesis and the subsequent conversion of OAA to oxalate and acetate (Fig. 4b, c). In addition to transcriptional regulation, PEPC activity is also regulated by phosphorylation catalyzed by phosphoenolpyruvate carboxylase kinase (PEPCK) [20]. PEPC phosphorylation is abolished in the pepc3 mutant of Arabidopsis under salt stress [20], suggesting that PEPC3 might be the target protein of PEPCKs. The significant increases in the expression levels of two PEPC1 genes and the phosphorylation level of PEPC3–1 also suggested the phosphorylation regulation of PEPC3–1 by PEPCK1s (Fig. 4b, c). Collectively, the above results suggest that the OAA pathway is the main pathway for oxalate and acetate biosynthesis under alkali stress. However, notably, the glycolate/glyoxylate pathway might also be modified in grapevine roots under alkali stress, as indicated by the significant increase in the phosphorylation level of aconitate hydratase (aconitase), a key protein in the glycolate/glyoxylate pathway [30].

H+ extrusion and the transport of organic acid anions across the plasma membrane are controlled by PM H+-ATPases, providing energy by creating an electrochemical proton gradient for transporters [31]. The PM H+-ATPase is important for the root proton-secretion adaptation to alkaline stress [15]; high PM H+-ATPase activity and proton secretion have been shown to enhance plant tolerance to alkaline stress [32], while lower PM H+-ATPase activity and proton secretion result in sensitivity to alkaline stress [33]. PM H+-ATPase activity is tightly regulated by phosphorylation at several N- and C-terminal residues, especially upon exposure to various environmental stimuli [34]. The significant increase in the phosphorylation levels showed that the two ATPases 4 are the key proton pumps in grapevine roots under alkali stress (Fig. 4b, Table 1). The ATPases 4 from Arabidopsis (AHAA4) and tobacco (PMA4) plants are also reported to be involved in salt tolerance [35, 36]. Therefore, ATPase 4 might function in plant tolerance to alkali and salt stress.

In contrast, the specific transporter of oxalate remains unclear. Nevertheless, some experiments have indicated the role of ABC transporters in transporting organic acids in plants. Four ABC transporters (ABCG11, ABCG21, ABCA2, and ABCB21) were considered to be potential involved in oxalate and/or citrate secretion under Al stress in Grain amaranth roots [10]. AtPDR6, belonging to the ABCG subfamily of the ABC transporter family, is involved in the root extrusion of organic acids, including succinate, fumarate and malate [37]. In this study, the expression levels of five ABC transporters, particularly ABCB19–1, were substantially increased by NaHCO3 treatment (Fig. 4b, Additional file 1: Table S1), suggesting their association with organic acid secretion. In particular, the expression of ABCB19–1 was well correlated with the oxalate content in roots (Fig. 4c, Fig. 1a), suggesting its role in transporting oxalate. Additionally, two ABC transporters, ABCB19–2 and PDR12, were significantly phosphorylated (Table 1), and PDR12 has been reported to mediate the extrusion of water-soluble carboxylate anions in yeast [38]; therefore, ABCB19–2 and PDR12 might participate in the transport of organic acids. However, the detailed function of the above transporters must be characterized in future studies.

ALMTs are found throughout plant genomes and are involved in a range of distinct functions in different cell types. ALMT1 functions in mediating organic acid secretion in the Al-tolerance response in many plant species [39]. In contrast, the ALMT2 transporter mediates an Al-independent electrogenic transport of organic anions, such as malate and citrate, across the plasma membrane in wheat [40]. The upregulation of two ALMT2s (Fig. 4b, c) and the increased content of malate implied that ALMT2 transporters mediate the salt- and alkali-induced malate extrusion in vine roots. Notably, a large increase in malate secretion was not accompanied by its accumulation under salt and alkali stress (Fig. 1b, h). A similar phenomenon was also found in other plants under Al stress [22, 41]. Therefore, it is suggested that malate metabolism is not a limiting factor but rather that transporters are more important for its secretion under alkali stress.

The signaling pathway mediating alkali stress-induced organic acid secretion

Ethylene is an important signaling molecule mediating numerous important biological processes, including responses to abiotic stresses [42]. In this study, the application of inhibitors related to ethylene biosynthesis and perception indicated the necessity of ethylene in organic acid secretion under alkaline stress (Fig. 6c, d). However, the exogenous application or endogenous overproduction of ethylene substantially inhibited H+-ATPase activity and H+ efflux in rice roots under alkali stress [17]; additionally, ethylene production was reduced with NaHCO3 treatment (Fig. 6a). Similarly, transgenic tobacco plants with poor ethylene
biosynthesis exhibited elevated salt tolerance, while rice plants treated with ethylene exhibited salt hypersensitivity [43, 44]. Therefore, the role of ethylene might be concentration-dependent [42, 45], and low-concentration ethylene might be necessary to mediate NaHCO3-induced organic acid secretion. Ethylene biosynthesis is primarily regulated by ACS and ACO at the transcriptional and posttranslational levels [46, 47]. When grapevines were subjected to alkali stress, the decline in ethylene production was accompanied (Fig. 6a) by the decreased expression of ACO1 (Fig. 4c) and phosphorylation levels of ACO4 and ACO11 (Table 1) but the increased expression of ACS3 and ACS7 (Fig. 4c), suggesting the key role of ACOs in regulating ethylene synthesis under alkali stress. Additionally, EIN2, a central regulator of ethylene signaling [48], controls the transduction of the ethylene signal from the ER membrane to the nucleus in Arabidopsis [48], and its phosphorylation inhibited ethylene signaling (Table 1; 48). After the signal cascade mediated by EIN5, EIN6, EIN3 and others, ethylene signals are delivered to ethylene responsive factors (ERFs), the last downstream components of the ethylene signaling pathway, which lead to the regulation of ethylene controlled gene expression [43]. Here, the changes in the expression of a large amount of ERFs may correspond to the different biological processes regulated by ethylene under alkali stress.

Auxin has been reported to control root apoplastic acidification, to enhance the AI-induced exudation of citrate and to promote the phosphorylation of PM H+-ATPases [13, 45, 49]. Additionally, PIN2 (an auxin efflux transporter) activates plasma membrane H+-ATPases to release protons, which is necessary for the adaptation of Arabidopsis to alkali stress [15]. Therefore, auxin plays a key role in regulating organic acid secretion under alkali stress. A recent study found that endogenous auxin controls apoplastic acidification; however, an endogenous increase in the overexpression of auxin biosynthesis gene or exogenous increase in the auxin level induces a transient alkalization [45], which is similar to the role of ethylene discussed above. Here, NaHCO3 treatment induced a decrease in IAA, but NPA treatment reduced organic acid secretion (Fig. 6b-d), suggesting that the fine-tuning of IAA biosynthesis may be essential for the regulation of organic acid secretion. Additionally, the genes of IAA12 and GHB3 as IAA repressors [45, 50] were also substantially upregulated (Fig. 4c), probably reducing IAA synthesis and signaling. Small auxin-up RNA (SAUR) genes represent the largest family of auxin-responsive genes and participate in auxin-mediated PM H+-ATPase activation [45, 51]. The large increase in the expression of SAUR32 and SAUR40 suggests that they likely regulate organic acid secretion in response to auxin in vine roots. Particularly, it has been demonstrated that auxin mediates ethylene signaling to control root growth [52]. Therefore, we inferred that ethylene most likely regulates organic acid secretion through auxin signaling in vine roots under alkali stress.

On the other hand, the cell membrane harbors hundreds of different receptor kinases that receive environmental signals at the receptor domain on the extracellular side of the membrane and convert these signals into cellular responses via an intracellular protein kinase domain [34]. The PM H+-ATPase was reported to interact with multiple such receptor kinases [53]. The receptor kinase-mediated control of primary active proton pumping at the plasma membrane, e.g., PSY1R, increases proton efflux from roots by interacting with and phosphorylating AHA2/AHA1 [54]. In this study, the high-level expression and/or phosphorylation of the 11 plasma membrane-located receptor kinases might participate in the regulation of ATPases. Although the exact roles of candidate genes remain to be examined, our data provide a platform for further functional analyses of these genes.

Conclusion
Oxalate was the primary organic acid synthesized and secreted by vine roots under NaHCO3 stress. The OAA pathway, including two PEPC3s and PEPCK1s, plays a key role in oxalate synthesis. The secretion of organic acids and H+ were controlled by PM H+-ATPases, and two phosphorylated PM ATPases 4 were the main proton pumps under NaHCO3 stress. Additionally, ABCB19–2 and PDR12 might participate in the transport of oxalate and other organic acids. Low-concentration ethylene mediates NaHCO3-induced organic acid secretion, and IAA also participates in this process.

Methods
Determination of organic acids in grapevine roots and root exudate solutions
Healthy apical growth tips of A15 vines were removed in early summer to establish grapevine in vitro shoot cultures. The shoot cultures were subcultured on Murashige and Skoog medium containing 3% (w/v) sucrose, 7 g. L−1 agar and 0.2 mg. L−1 IBA. Five-week-old grapevine in vitro shoot cultures were transferred to glass bottles with a 10-cm height and 5-cm diameter. The vines were treated with 50 ml water (pH7.0) as a control, 75 mM NaCl (pH7.0) and NaHCO3 (pH8.7). Each glass bottle was provided sufficient oxygen with an oxygen machine (SenSen Group, China). The vines were grown in a controlled-environment growth cabinet at 25 °C, a 16-h photoperiod and a light intensity of 600 μmol/m2/s. At different time points after different treatments, the roots were collected and immediately frozen in liquid nitrogen for organic acid extraction. The treatment...
solution was collected and evaporated to dryness with a rotary evaporator, and the residue was dissolved in 1 ml double distilled water. The filtrate, which was passed through a 0.45-μm filter, was used for organic acid determination. The extraction of organic acids from the roots and their determination for root extract and treatment solutions were performed using a capillary electrophoresis system (Beckman P/ACE, Palo Alto, CA) as described in our previous study [55].

H+ secretion test
H+ secretion was detected according to a previously described method [56]. Five-week-old grapevine A15 in vitro shoot cultures were treated with water (pH 7.0), 75 mM NaCl (pH 7.0), 75 mM NaHCO3 (pH 8.7), and 75 mM NaHCO3 plus 0.1 mM Na3VO3 for 6 h. Then, the roots were rinsed, carefully spread in Petri dishes and covered by solid medium (pH 5.8) which consisted of 0.006% bromocresol purple (pH indicator, discoloration range of 5.2–6.8), 1 mmol L−1 CaSO4, 2.5 mmol L−1 K2SO4 and 0.8% agar. The vines were grown in a controlled-environment growth cabinet at 25 °C and continuous illumination at 400 μmol/m2/s light intensity.

Measurement of H+-ATPase activity and H+ flux in roots
Plant materials were the same as those described in the organic acid determination Section. The extraction of plasma membrane (PM) protein and activity determinations of PM H+-ATPase of the root tips were conducted according to the method of Yan et al. (2002) [56]. The activity of H+-ATPase was determined by the Pi amount between two samples were screened using a false discovery rate < 0.05 and absolute log2 (fold change) ≥1 as the threshold. Three biological replicates were generated for the control and NaHCO3 treatment.

qRT-PCR was performed using SYBR Green Master-Mix (SYBR Premix EX Taq TM, Dalian, China) on a Bio-Rad iQ5 (Hercules, CA, United States) instrument, and the primers are listed in Additional file 3: Table S3.

Phosphopeptide and phosphoprotein identification and quantification
Proteins from grapevines were extracted and quantified with the BCA kit (Beyotime, Beijing, China). After trypsin digestion, the peptide mixture was desalted on a Strata X C18 SPE column (Phenomenex, Torrance, CA, USA). Each peptide was vacuum-dried and reconstituted in 0.5 M TEAB (Sigma, USA). The peptide mixture was labeled using TMT kit (Thermo Fisher Scientific, Torrance, CA, USA) according to the manufacturer’s introduction. The TMT labeled peptides were fractionated by high pH reverse-phase HPLC and concentrated by vacuum centrifugation. The phosphopeptides were enriched using IMAC microspheres, and they were then eluted with elution buffer, followed by lyophilization and LC-MS/MS analysis. The phosphopeptides were dissolved in 0.1% formic acid (solvent A) and loaded onto a C18-reversed phase column (15-cm length, 75 μm i.d., packed in-house) and separated with a linear gradient of solvent B (0.1% formic acid in 98% acetonitrile) at a constant flow rate of 0.4 μL/min on an EASY-nLC 1000 UPLC system (Thermo). The peptides were eluted with a gradient of 6 to 23% solvent B for 26 min, 23 to 35% solvent B for 8 min, and 80% solvent B for 6 min. The peptides were detected and identified by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC, which was supported by Jingjie PTM BioLabs (Hangzhou, China). MS/MS data were searched using a Maxquant search engine (v1.5.2.8) against the Vitis vinifera proteome concatenated with reverse decoy database. The parameters in Maxquant searches were as follows: max missing cleavage of Trypsin/P, 2; peptide mass tolerance, 20 ppm in the first search and 5 ppm in the main search; MS/MS tolerance, 0.02 Da; fixed modification, carbamidomethyl on Cys, i.e., [2019] 19:383
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Cys; variable modification, oxidations on Met. FDR was adjusted to < 1%.

**IAA extraction and determination**

IAA extractions were performed according to our previous study [57]. Separation and quantification of IAA were carried out using a Scientific Ultimate 3000 HPLC system (Thermo, San Jose, CA, USA) coupled to a TSQ Quantum Access MAX system (Thermo, San Jose, CA, USA). HPLC separation was performed using a Thermof Scientific Hypersil Gold column (50 × 2.1 mm, 1.9 μm). The injection volume was 10 μL. The mobile phase consisted of 0.5% acetic acid in water (A) and methanol (B) with the following gradient at a flow rate of 1.0 mL min⁻¹: 0–0.5 min, 0–20% B; 0.5–3.0 min, 20–90% B; 3.0–6.5 min, 90% B; 6.5–10.0 min, 90–20% B; 10.0–15.0 min, 20% B. Detection and quantification of IAA were performed using the ESI negative mode. The parameters were set as follows: parent mass by charge (m/z) of 263.1, daughter mass by charge (m/z) of 153.0, and a collision energy of 14 eV.

**Determination of the ethylene production rate**

The ethylene production rate was measured using a GC-9A gas chromatograph (Shimadzu, Japan) equipped with a GDX-502 column and a flame ionization detector. The vine roots were enclosed in a 5-mL centrifuge tube with sealing film and incubated at 25 °C for 3 h. Five milliliters of the headspace gas was withdrawn from each tube through the septum stopper using a gas-tight syringe and assayed.

**Statistical analyses**

Statistical analysis was performed with the SPSS (V19.0) statistical software package. A one-way analysis of variance followed by Duncan’s multiple range test was employed.

**Additional files**

**Additional file 1:** Table S1. RNA-Seq profiles of the control and NaHCO₃-treated vine roots. Unigenes differentially expressed between two samples were screened using a false discovery rate < 0.05 and absolute log₂ (fold changes) ≥ 1 as the threshold. (XLSX 1337 kb)

**Additional file 2:** Table S2. Analysis of phosphopeptide changes in grapevine roots exposed to NaHCO₃ stress. The significantly altered phosphopeptides between two samples were screened using fold changes ≥1.5 or ≤ 0.67 (P < 0.05). (XLSX 96 kb)

**Additional file 3:** Table S3. Primer sequences for real-time quantitative RT-PCR. Gene ID is derived from the grape genome (http://genomes.criib.ucrbi.uniud.it/grape/). (DOCX 17 kb)

**Abbreviations**

1-MCP: 1-methylcyclopropene; ABCB19: ATP-binding cassette B19; ACC: 1-aminoacyclopropane-1-carboxylic acid; ACH: Aconitate hydratase; ACO: 1-aminoacyclopropane-1-carboxylic acid oxidase; ACS: 1-aminoacyclopropane-1-carboxylic acid synthase; ALMT: Aluminum-activated malate transporter; AVG: Aminoethoxyvinylglycine; DAT: Days after treatment; DEG: Differentially expressed gene; HAT: Hours after treatment; IAA: Indoleacetic acid; ME: Malic enzyme; NPA: 1-N-naphthylphthalamic acid; OCL: Oxalate-CoA ligase; PDR12: Pleiotropic drug resistance 12; PEPC: Phosphoenolpyruvate carboxylase; PEPPC: PEPC kinase; PWM: Plasma membrane; qRT-PCR: quantitative real-time PCR; RPMK: Reads per kilobase of transcript per million mapped reads.

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Not applicable.

**Authors’ contributions**

YY and GX conceived and designed the research; GX, WM and SG performed the experiments; ZJ and QY analyzed the data; and YY wrote the manuscript. All authors read and approved the manuscript.

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**Availability of data and materials**

The mass spectrometry proteomics data have been deposited to the ProteomeXChange Consortium via the PRIDE partner repository with the dataset identifier PXD013746 (http://www.ebi.ac.uk/pride). Full RNA-Seq data were submitted to the sequence read archive (SRA) of NCBI under BioSample accessions SAMN11579694 and SAMN11579695 (https://www.ncbi.nlm.nih.gov/sra).

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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