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Research Paper

Effects of alkaline stress on organic acid metabolism in roots of grape hybrid rootstocks

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ABSTRACT

To elucidate the mechanism of organic acid metabolism in rootstocks under alkali stress, rootstocks A15 and A17 (*V. amurensis* cv. Zuoshan1 × SO4), both with strong alkaline resistance, and a control rootstock 1103P (*V. berlandieri* × *V. rupestris*) were used as materials. Tissue culture plants were treated with $NaHCO_3$ or Na_3VO_4 (H^+ − ATPase inhibitors). The pH value of the three rootstock rhizospheres decreased following $NaHCO_3$ stress. Oxalic acid was the main organic acid, and its secretion significantly increased after $NaHCO_3$ treatment. Oxalic acid secretion of A15 and A17 was significantly higher than that of 1103P before and after $NaHCO_3$ treatment. Oxalic acid content and secretion decreased in rootstock A15 under Na_3VO_4 + $NaHCO_3$ treatment. The changes in PEPC (phosphoenolpyruvate carboxylase) activity in A15 and the ICL (isocitrate lyase) activity in A17 were significantly increased. The relative expression of *VvICL* (isocitrate lyase gene) in A15 and *VvPEP* (phosphoenolpyruvate carboxylase gene) in A17 was significantly upregulated compared with the control. The relative expression of *VvPMA3* (H^+ −ATPase family gene) was higher in rootstock A15 and peaked after 24 h treatment. This suggests that the proton pump (*VvPMA3*) may play an important role in $NaHCO_3$ stress, through promotion of organic acid secretion in the roots to reduce $NaHCO_3$ stress.

1. Introduction

Soil salinization is an important environmental problem that restricts agricultural development and crop growth (Yang et al., 2011). High saline and alkaline soils account for approximately 36 million hm^2 of land in China (Wang et al., 2011), and approximately 1/3 of the total saline-alkaline land in Asian (Wang 1997). Northern arid, semi-arid and semi-humid areas hold the majority of salt and alkaline land in China, and the salt and alkaline land in Xinjiang accounts for about a third of the area of total cultivated land (Du et al., 2015). The composition of natural saline soil is complex, and most of the salinized soil is composed of NaCl, Na_2SO_4 and $NaHCO_3$; alkali stress is more harmful to plants (Li et al., 2010). Grape plants have strong salt tolerance compared with other fruit trees (Wang et al., 2000; Ma et al., 1997), but the salinity tolerance of *V. labrusca* rootstocks is generally lower than that of *V. vinifera* species. Most studies to date mainly focus on salt stress (Zhou et al., 2009; Qin et al., 2010; Fan et al., 2007); research on alkali stress is scarce (Yang et al., 2008).

Organic acids are considered as chelating agents of plant cells (Gibson and Sewell, 1992), and play an important role in the interaction between soil and microbes. Microbes promote soluble iron uptake from the soil (Johnson et al., 1994). Studies have shown that organic acid metabolism plays an important role in plant adaptation to alkali stress. Synthesis, accumulation, transport and secretion of organic acids is increased under alkali stress, (Wu and Fu, 2009; Rojas et al., 2012), suggesting roots secrete different types and amounts of organic acids to reduce damage under alkali stress. Organic acid accumulation may occur through complex and adaptive mechanisms in response to alkali stress, to reduce damage caused by pH and ion imbalance. However, the biochemical basis and metabolic regulation mechanism of organic acid accumulation in plants under alkaline stress are still unclear.

In our previous study, the $NaHCO_3$ salt tolerance of the F1 generation hybrid (*V. amurensis* cv. Zuoshan1 × SO4) with cold hardiness and phylloxera resistance was evaluated, and rootstock 1103P was used as the control. The previous results showed that A15 had strong alkali resistance, A17 had medium alkali resistance, and the alkali resistance of 1103P was weak. Therefore, in this experiment, the three rootstocks with different alkali resistance were used as materials to study the organic acid metabolism in roots under $NaHCO_3$ treatment, to reveal the mechanism of alkali resistance in rootstocks.

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2. Materials and methods

2.1. Materials

The alkaline resistant rootstocks A15 and A17 (*V. amurensis* cv. Zuoshan1 × SO4) and control rootstock 1103P (*V. rupestris* × *V. berlandieri*) were used as test materials.

2.2. H^+ secretion test

H^+ secretion was evaluated according to the methods of Yan et al. (2002). One-month old tissue culture seedlings of A15, A17 and 1103P were treated with 50 mmol L^{-1} $NaHCO_3$ and the control was treated with Holland nutrient solution. After 12 h $NaHCO_3$ treatment, the roots were rinsed and put in petri dishes. The solid medium (pH 5.8) consisted of 0.006% bromocresol purple, 1 mmol L^{-1} $CaSO_4$, 2.5 mmol L^{-1} K_2SO_4 and 0.8% agar. The medium was heated until melted and then cooled to 40 °C in a water bath. The culture medium was then poured into the petri dishes until the roots were soaked. After the culture medium solidified, the plants were placed in the dark for 24 h at 28 °C. The pH of the culture medium around the roots was measured using a pH indicator (bromocresol purple, discoloration range of 5.2–6.8) to qualitatively evaluate H^+ secretion in the roots.

2.3. Organic acid analysis of the root exudate solutions

One-month old tissue culture seedlings of similar size were used. The medium was washed off gently, and the seedlings were put in glass bottles (10 cm high and 6 cm diameter), one plant per bottle. The plants were treated with control (Holland nutrient solution), 0.1 mM Na_3VO_4, 100 mM $NaHCO_3$ and 100 mM $NaHCO_3$ + 0.1 mM Na_3VO_4. There were five plants per treatment. The plants were grown in a controlled-environment growth cabinet with a 14 h/23 °C day at a light intensity of 400 μmol m^{-2} s^{-1} and 10 h/20 °C night regime. During the treatment, each glass bottle was provided with sufficient oxygen with an oxygen machine (941, SenSen Group, China). After 2 days of treatment, the liquid solution in the glass bottle was collected and evaporated to dryness with a rotary evaporator (SHB-III circulating water type multi-purpose vacuum pump), and the residue was then dissolved in 1 ml water and passed through a 0.45 μm filter (Jinlong, China). The samples were analyzed using an HPLC (Waters 515) according to Ma et al. (1997). Detection (Waters 2487) with 0.6 AUFS sensitivity was at 210 nm.

2.4. Root organic acid analysis

Plant materials and treatment were the same as that used in Section 2.3. The roots were homogenized in a cold 1.5 ml phosphate buffer and then passed through a 0.45 μm filter. The organic acid content of the roots was analyzed according to that described in Section 2.3.

2.5. Response of related enzyme of oxalate metabolism under alkali stress

Tissue culture seedlings were placed in glass bottles (10 cm high and 6 cm diameter), one plant per bottle. The plants were treated with control (Hoaglang nutrient solution) or 100 mM $NaHCO_3$, 5 individuals for each treatment. The activities of oxalate metabolism related enzymes were determined at 0 h, 12 h, 24 h, 48 h and 96 h after $NaHCO_3$ treatment, and the enzyme activity of isocitrate lyase (ICL) and phosphoenolpyruvate carboxylase (PEPC) were determined by enzyme-linked immunosorbent assay (ELISA). The expression of the isocitrate lyase gene (*VvICL*), the phosphoenolpyruvate oxidase gene (*VvPEP*) and the proton pump gene (*VvPMA*) was analyzed. The expression of *VvICL* and *VvPEP* was determined 0 h, 3 h, 6 h, 12 h, 48 h after $NaHCO_3$ treatment, and the expression of *VvPMA* was determined at 0 h, 6 h, 12 h, 24 h and 48 h after $NaHCO_3$ treatment.

Table 1
Primer pairs for real-time PCR.

gene	primer sequence (5′-3′)
VvUBIRQ	GTGGTATTATTGAGCCATCCTT AACCTCCAATCCAGTTATCTAC
VvICL	AATCTGGATGGAGACCGCAAG CTGTTCATCCGTCATTCCT
VvPEP	TAGCCGTCCATCAAAGCGTAA TCAATGGCATATTTAAAAGCT
VvPMA1α	TAGAAGAGAAAAAGGAAAGCA CAACAAAGTCCTGCCAATC
VvPMA1β	GTTGTCTGATTGTGTGTTTGG TGCCAGCATTGTTTTCTTC
VvPMA3	GAAAGAGAAGGAGAGAAACAAG GGAAATAATAGACGGCAAAC

Total RNA was extracted from root tissues using the Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and digested with DNase I at 37 °C for 15 min to remove contaminating DNA. The RNA was cleaned using a RNeasy Kit (Qiagen, Hilden, Germany) and the quantities and qualities were determined by spectrophotometry and 1% formaldehyde denaturing gel electrophoresis, respectively. The samples with bright bands of ribosomal 28S to 18S RNA in a ratio > 1.5:1 were used for microarray analysis. Primers were designed according to the principle of fluorescence quantitative PCR primer design (Sangon Biotech, China), and the primer sequences are shown in Table 1. The internal reference gene was *VvUBIRQ*. RNA was used to synthesize cDNA (TaKaRa, Japan) according to manufacturer's instructions and yielded 20 μl of reverse transcription mix. The reverse transcription mix was then diluted to a final volume of 40 μl, and 1 μl was used for the qRT-PCR experiments. The ChamQ SYBR qPCR Master Mix was produced by Vazyme, China. Reactions were carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with Power SYBR-Green PCR Master Mix (Applied Biosystems) and gene-specific primers. Cycling conditions were: initial denaturation (94 °C, 10 min) followed by 40 cycles of denaturation (94 °C, 30 s), annealing, and extension (60 °C, 1 min). The relative value for expression level of each gene was calculated by the $2^{-\Delta\Delta CT}$ method based on Livak and Schmittgen (2001).

2.6. Data processing

Microsoft Excel software was used to process data and produce the figures. DPS software LSD method was used to calculate significance.

3. Results

3.1. Effects of $NaHCO_3$ treatment on root H^+ secretion from different rootstocks

A15, A17 and 1103P were treated with $NaHCO_3$ for 12 h and then placed in solid medium, as shown in Fig. 1. Color change occurred around the roots to indicate changes in pH. The pH value of the medium around the roots decreased, indicating that the roots secreted an acidic substance following $NaHCO_3$ treatment. The yellow area around the A15 rootstock was the largest, indicating acidic secretion of the A15 rootstock was highest following $NaHCO_3$ treatment. Rootstock A17 showed less acidic secretion, while the acidic secretion of 1103P was the least.

3.2. Effect of $NaHCO_3$ on the secretion of organic acids from different rootstocks

Seven organic acids were detected, namely oxalic acid, tartaric acid,