Overexpression of Vvcyp86a1 Improves Salt Tolerance in Arabidopsis Thaliana during Germination and Seedling Stages

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Abstract: Grape (vitis vinifera L.) is a fruit tree which is considered to be a horticultural crop of major economic importance. The salt tolerance of grape is relatively high. Suberin in grape roots play important roles in salt stress response of grape. CYP86A1 plays a crucial role in the ω-hydroxylation of fatty acids, which are considered to be the main components of aliphatic domains. While, the function and the regulatory mechanism of CYP86A1 in response to salt stress in grape was not revealed. In the present study, VvCYP86A1 was isolated from vitis vinifera L. The expression of VvCYP86A1 was significantly inducued by salt stress, particularly in roots. Overexpression of VvCYP86A1 increased the tolerance to salt stress in Arabidopsis during both germination stage and seedling stage. The improvement in salt tolerance was the result of the combined contributions of multiple mechanisms including accumulating osmotic regulating substances and maintaining membrane stability.

1. Introduction

Salt stress is one of the major environmental stresses worldwide. It has been reported that 20% of the 230 million hectares of irrigated farmland are affected by salt with an increasing proportion every year [1]. Planting salt-tolerant plants on saline-alkali land is considered to be the most economical and effective way to improve and utilize saline-alkali land. However, the high ion concentration of saline-alkali land would lead to osmotic shock and ionic toxicity which both seriously reduce the growth of plants. Therefore, maintaining a low cytoplasmic concentration of toxic ion (such as Na^+ and Cl^-) is quite important for plant survival under salt stress[2].

Grape (*vitis vinifera L.*) is a fruit tree which is considered to be a fruit tree of major economic importance [3].The salt tolerance of grape is relatively high[4-6]. However, genetic variability exists among grape genotypes with variable sensitivity to salt stress [6-8].

It has been reported that salt exclusion, which characterized by low Na^+ content in leaves and relative high Na^+ content in roots, plays important roles in response to salt stress in grape [9]. Under salt stress, the roots endodermal cells of grapevines showed greater K^+ selectivity and enhanced exclusion of Na^+ and Cl^-[10]. The existence of root apoplast barrier and the function of ion transporters may both contribute to this [11,12]. Apoplastic barriers is consisted of Casparian bands (CB) and suberin lamellae (SL) in the endo- and exodermis [13-15]. Apoplastic transpiration bypass flow from roots to shoots of water and solutes can be blocked by root apoplastic barriers [15-18]. This effect is believed to play a vital role in the salt exclusion of plants. SL is an important part of the apoplast barrier. The suberization of apoplast barriers under salt stress has been reported in a variety of plants [13,19], which may contribute to the salt stress resistance.

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Suberin can be chemically described as an biopolyester mainly comprised of ω-hydroxyacidsand, α,ω-dicarboxylicacids (diacids) and lower amounts of fatty acids and alcohols [13,20,21]. The hydroxylation of fatty acids in plants is catalyzed by specific cytochrome P450 subfamilies, particularly *CYP86A*, *CYP86B* and *CYP94A*[13,22,23].

Additionally, it has been reported that changes in CYP86A encoding genes could lead to changes in components of suberin and environmental responses. C16 and C18 ω-hydroxy acid and α, ω-dioic acid in the tuber cortex were reduced by 70%~90% in cyp86a3 in potato [24]. For Arabidopsis thaliana, in the *CYP86A1* mutant (horst) the total amount of suberin in the root was reduced by 60%, and C16 and C18 ω-hydroxy acids and α, ω-diacids showed the most significant reduction [25]. The expression of *CYP86A1* was also closely related to the salt stress response in plants. The *CYP86A1* mutant in Arabidopsis thaliana exhibited a salt-sensitive with higher Na^+ accumulation and lower K^+ accumulation in shoots [26].

Although there were numerous studies on the function of *CYP86A1*, the function and the molecular mechanism of it in response to salt stress of grape were still unknown. In the present study, the *VvCYP86A11* gene was cloned and transformed into Arabidopsis. The function of it during germination stage under salt stress was also investigated. Results of this study might provide information for understanding the mechanism of *VvCYP86A1* in response to salt tolerance.

2. Materials and Methods

2.1 Plant Materials, Cultivation, and Treatment

The grapestocks were grown in tissue culture bottle containing MS medium adding 200 ug/L Indole-3-Butytric acid (IBA) under 26 ± 2 ℃ with a 16/8 h (light/dark) photoperiod and lightintensity of about 45 ± 5 μmol m−2 s−1. When grape rootstock tissue culture seedlings were rooted , some seedlings were treated with 0, 50 mM NaCl for 48 h. Roots, stems and leaves from the treated seedlings were stored at -80 °C for determination of the expression level of *CYP86A1* using forward (5'-TACTCCATTCACTGACTA-3') and reverse (5'-ATAGAACCAGAGGACATA-3') primers. Vitis vinifera L's housekeeping gene actin was used as an internal standard.

Salt tolerance identification was carried out using Arabidopsis Col-0 wild type (WT) and *VvCYP86A1* overexpression lines. *VvCYP86A1* was overexpressed in Arabidopsis promoted by CaMV 35S, and generated three T3 homozygous transgenic lines (OE2, OE3 and OE4). Dry seeds were stored at 4℃.

The sanitized seeds were sown on 1/2 MS medium with 0, 75, 100 or 125 mM NaCl. Plants were incubated at 23 ± 2℃ under a 10/14 h (light/dark) photoperiod and light intensity of 200 ± 50 μmol m^{-2} s^{-1}.

For seedling-stage experiment, the Arabidopsis seeds were plated in 10 cm × 10 cm × 10 cm red square plastic pots filled with nutrient soil at 23 ±2 °C under a 16/8 h (light/dark) photoperiod and light intensity of 200 ± 50 μmol m^{-2} s^{-1}. At the four-leaf stage, seedlings were treated with Hoagland solution with 0 or 100 mM NaCl for 14 days. The NaCl concentration increased stepwise towards the final concentrations by 50 mM each day.

2.2 Cloning and Sequencing of Vvcyp86a1

The full-length *VvCYP86A1* was determined from the National Center for Biotechnology Information reference genome (https://www.ncbi.nlm.nih.gov/nuccore/XM_0106 52629). We obtained two sequences of *VvCYP86A1* using forward (5'-CGCGGATCCGGCACAACAGTCTACTCCATT-3') and reverse (5'-CCGGAATTCCATGGACAACTATA CCCTC4.4. Analysis of Seed Germination Indicators

2.3 Analysis of Seed Germination Indicators

The germination rate (GR),germination energy (GE) and germination index (GI) of WT and overexpression lines were measured. GR, GE and GI were calculated via the following formulae:

$$GR=\frac{G7}{T}\times 100\%$$

$$GE=\frac{\sum G3}{T}\times 100\%$$

$$GI=\sum\frac{Gt}{Dt}$$

where Gt is the number of seeds germinated on the tth day, T is the total number of seeds, Dt is the number of days up to the tth day.GT-3') primers.

2.4 Analysis of Weight of Arabidopsis during Seedling Stage

The phenotype, fresh weight (FW) and dry weight (DW) of different lines during seedling stage was measured after NaCl treatment for 14 days.

2.5 Determination of Malondialdehyde Content and Soluble Proline Content

Malondialdehyde content and soluble proline content were measured as described previously [6] using 0.1 g of fresh leaf material. Three replicates were included for each treatment.

3. Results

3.1 Expression Level of Vvcyp86a1 in Two Genotypes of Vitis Vinifera l.

The relative transcript levels of *VvCYP86A* in leaves, roots and stalk of Crimson Seedless and 1103P under treatment of 0 or 50 mM NaCl treatment were detected by qRT-PCR. The results showed that in Crimson Seedless, the expression of *CYP86A1* in each tissue of the control group was relatively low. While, the expression level of it decreased slightly in leaves but increased by 5 times in stalk under salt stress. To our surprise, the expression level of *CYP86A1* was induced to 27 times by salt stress in roots. In 1103P, the expression level of *CYP86A1* of control group in leaves and stalk was also relatively low. However, expression level in roots was about 7 times higher than that in the other two tissues. Under salt stress, the expression of *CYP86A1* decreased slightly in leaves and stalk. In contrast, it in roots was significantly up-regulated although it was far less than that of Crimson Seedless .

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3.3 Identification of Vvcyp86a1 Overexpression Lines

T3 homozygous transgenic lines(OE2, OE3 and OE4)were obtained.

3.4 Effects of Salt Stress on Different Arabidopsis Lines during Germination Stage

The germination of seeds is the basis of plant growth and development. According to the results shown in Figure 1, there is no significant difference in the growth of all lines without NaCl treatment. While, the growth of each line was inhibited by salt treatment. The effect of salt stress on growth of OE4 was similar to WT. However, the two lines OE2 and OE3 showed significant salt tolerance phenotypes.

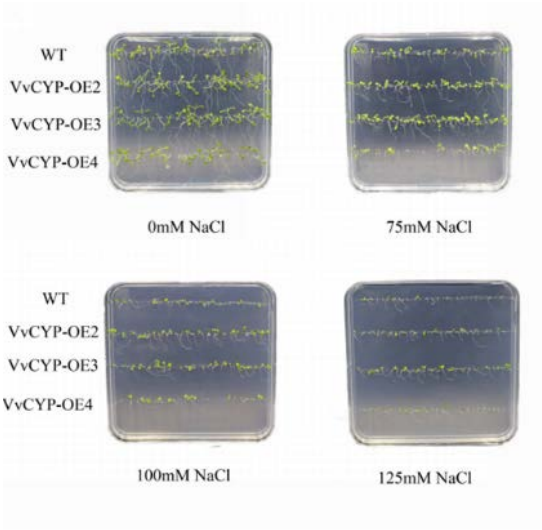

Fig.1 The Phenotype of Wt and Transgenic Arabidopsis Lines under Different Nacl Concentrations during Germination Stage.

In terms of germination indicators, the GR of WT and the OE lines were roughly the same without salt treatment. However, with the increase of treated NaCl concentration, the GR of different lines gradually decreased (Figure 2). The reduction of GR in WT is more significant than all other lines. When NaCl concentration reached 125 mM, the GR of OE2, OE3, and OE4 were 69%, 72% and 66% higher than that of WT. GE reflects the germination activity of seeds. According to our results, GE in all lines reduced under salt stress and the reduction of WT was much more significant than that of OE lines. Similarly, WT also had a stronger reduction in GI than that of other lines (Figure 2). These results indicated that overexpression of CYP86A1 could protect plants from salt inhibition during the germination stage.

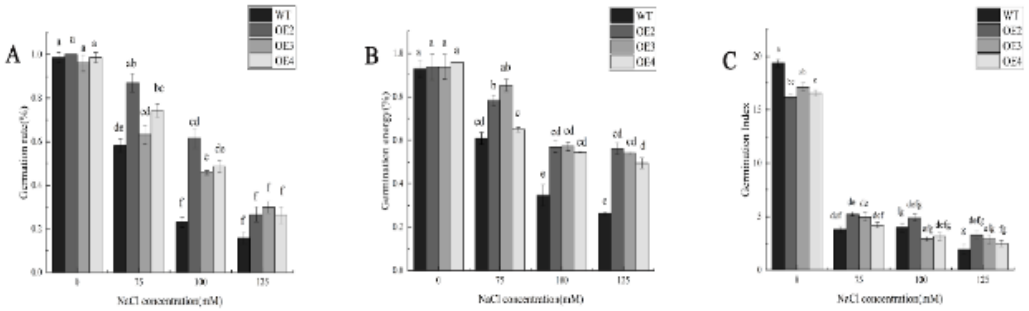

Figure 2. The germination indicators of WT, transgenic Arabidopsis lines under different NaCl concentrations. Bars with the different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

3.5 Effects of Salt Stress on Different Arabidopsis Lines during Seedling Stage

Without NaCl treatment, all lines grew well. Plant size of OE2 and OE3 were little smaller than WT and OE4. After treated with 100 mM NaCl for 14 days, the growth of both WT and OE lines were inhibited. This inhibitory effect was much more significant in WT (Figure 3A).

Change in fresh weight is an important physiological index to measure the ability of plants to resist salt stress. As shown in Figure 6, fresh weight of shoots and roots were decreased in all the lines, particularly in WT. Shoots fresh weight in WT, OE2, OE3, and OE4 decreased by 14.3%, 7.6%, 12.5%, and 8.2%, respectively (Figure 3B). As roots are the organs directly contacting saline environment, salt stress has a greater impact on the fresh weight of roots. The fresh weight of roots in WT decreased by 20.1%, and it in OE2, OE3 and OE4 lines decreased by 18.4%, 16.7 and 16.5%, respectively (Figure 3C). The results indicated that the overexpression *VvCYP86A1* broght Arabidopsis a stronger ability to resist salt stress.

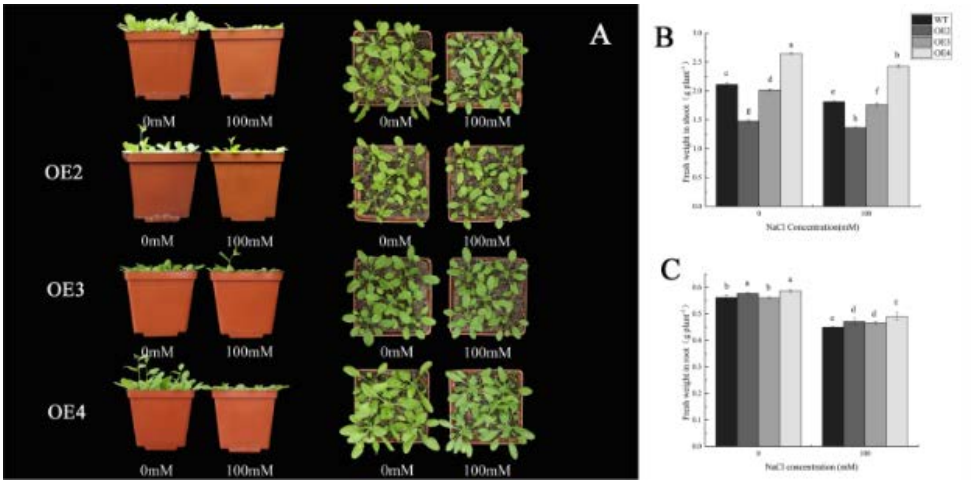

Fig.3 The Phenotype (a) and Fresh Weigh in Shoot (B) and Root (C) of Wt and Transgenic Arabidopsis Lines under 0,100mm Nacl Concentrations during Seedling Stage.

3.6 Changs in Proline and Mda Content under Salt Stress during Seedling Stage

Under salt stress, the content of MDA increased significantly in WT and OE4 by 45.4% and 17.3%, respectively. However, the MDA content did not change significantly in OE2 and OE3 (Figure 4A). The proline content increased in all lines after treated with NaCl. Among them, the change of proline in OE3 was not significant. The proline content in WT, OE2, and OE4 increased by 124.5%, 42.4% and 401.2%, respectively (Figure 4B).

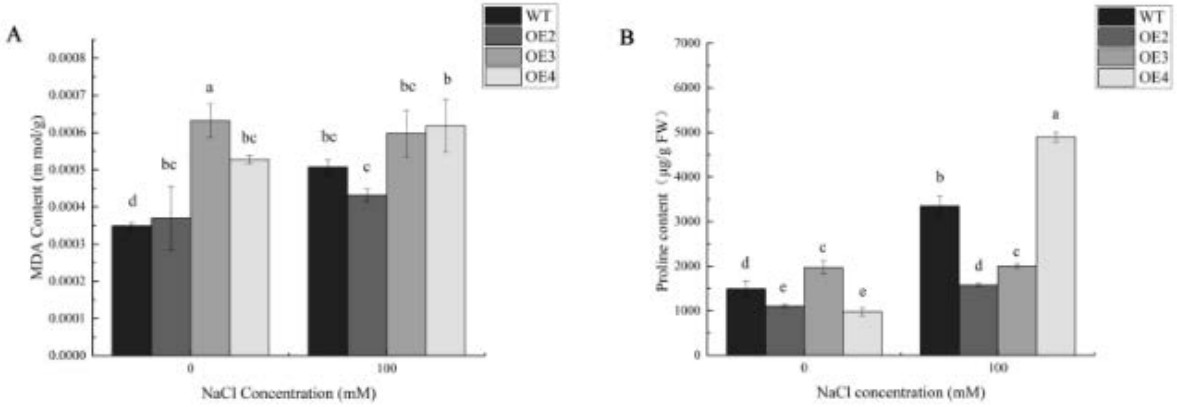

Figure 4. MDA (A) and Proline content content(B) of WT and transgenic overexpression lines of Arabidopsis treated with 0 and 100 mM NaCl for 14 d. Bars with the different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

4. Discussion

Grape (*Vitis vinifera L.*) which has high economic and nutritional value are widely planted worldwide. While, the development of viticulture still faces many problems including soil salinization[5,27]. Some vineyards are located in semiarid area which usually accompanied by soil salinization.uthors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

Grapes have a relatively high tolerance to salt stress [19,28]. Although researches on salt tolerance of grapes has received extensive attention [29,30]. However, there were relatively few studies on the molecular mechanism of salt response in grape and the use of molecular means to improve the salt tolerance of it. It has been reported that salt tolerance of grape was related to the characteristics of salt exclusion, which may limit the transport of Na^+ from root to shoot[10,28].Under salt tolerance, grape can accumulate Cl^- in the roots rather than the shoots[5]. Storey et al. (2003) found that the

root endoderm cells of grape exhibited significant Na^+ and Cl^- exclusion under salt stress[10], which was consistant to some studies in crops [12,15,17,25]. Additionally, the ability of salt exclusion was also considered to be an important indicator to measure the level of salt tolerance and screen salt-tolerant plants[27]. However, the physiological and molecular mechanisms of salt exclusion in grape were still unclear.

SL plays a pivotal role in salt exclusion [26]. Additionally, studies have shown that suberin content of salt-tolerant plant species remained stable or even increased by salt stress. It in salt-sensitive species, however, decreased under salt stress [15,16]. The fuction of SL in blocking water and ions is mainly dependent on the the aliphatic domains[14,31]. *CYP86A1* plays a crucial role in the ω-hydroxylation of fatty acids, which are the main components of aliphatic domains [32].

According to our results, *VvCYP86A1* in 1103P and Crimson seedless were both up-regulated under salt stress and showed a root-specific expression pattern (Figure S1). This up-regulation was more significant in salt-tolerant varieties Crimson Seedless. This suggested that *VvCYP86A1* might be involved in the salt stress response by enhancing the synthesizing of suberin.

While, most studies of *CYP86A1* were focused on development[22,23,25,33], its function in salt response was almost unclear. To reveal the role of it in salt stress, we screened and identified the *VvCYP86A1* overexpression lines of Arabidopsis (OE2, OE3 and OE4) to determine the role of the gene in plant salt tolerance.

Seed germination is the start point and also the most critical stage in plant life [34].We observed that the growth and germination indicators of WT and OE lines were inhibited under 75, 100 and 125 mM NaCl treatments. The degree of inhibition in WT is much more severe than in OE lines, (Figure 1,2). These indicated that the overexpression of *VvCYP86A1* may improve salt tolerance during germination stage. In the experiment using plants of the seedling stage, we also got similar results to the germination stage. Under 100 mM NaCl, the phenotype and fresh weight of shoots and roots of WT and OE lines were suppressed to varying degrees. The degree of inhibition in WT was significantly higher than in OE lines (Figure 3). These results indicates that the overexpression of *VvCYP86A1* in the seedling stage can also improve the salt tolerance of plants to a certain extent.

Salinity often accompanied by oxidative stress. MDA, which is the product of membrane peroxidation, is considered to be an important indicator of membrane lipid peroxidation [35-37]. According to our results, MDA content increased much more significantly than that in OE lines under salt stress (Figure 4A). These findings are similar to those of Guo et al (2018)[38], suggesting that overexpression of VvCYP86A1 may play a positive regulatory role in salt and peroxide stress and decrease the extent of membrane peroxidation.

Typically, there is a strategy of plants to adapt to the environmental stress by accumulating compatible osmolytes [39,40]. Proline is considered to be an important osmolyte [41]. Additionally, proline play an important role in stableing structure of biological macromolecules, and regulating cellular redox potential [42]. In the current study, proline content in the WT, OE2 and OE4 increased significantly under salt stress (Figure 4B). While, in OE3, no significant change was observed in proline. This result was consistent with the results of OE2 in changes of MDA and growth phenotypes under salt stress during seedling stage (Figure 3A, 4A). It is speculated that the suberin lamellae in roots of OE2 was strengthened significantly in all the lines. This led to a better resistance to Na^+, so there is no obvious change in some salt tolerance index. However, considering the growth phenotype at the seedling stage, the plants of OE2 are smaller than other lines even under the control condition (Figure 3A). This may be due to the formation of a stronger suberin lamellae at the seedling stage, which slows down the transportation of nutrition through the apoplastic pathway.

5. Conclusions

In conclusion, we demonstrated that the expression of *VvCYP86A1* was activated by salt stress. Overexpression of it increased the tolerance to salt stress in Arabidopsis during both germination stage and seedling stage. The improvement in salt tolerance was the result of the combined contributions of multiple mechanisms including accumulating osmotic regulating substances and

maintaining membrane stability. However, the mechanism of suberin for increasing salt tolerance should be addressed in future studies.

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