Overexpression of Splicing Variants *VvPMA1β* from *Vitis Vinifera* L. Improve Salt Tolerance in *Arabidopsis Thaliana*

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Abstract. Plasma membrane H^+-ATPase (PMA) plays a very important role in regulating plant response to salt stress and decreasing Na^+/K^+ in cytosol. We found earlier that two splicing variants, *VvPMA1α* and *VvPMA1β*, were produced from *VvPMA1* gene in grape root plasma membrane under salt stress. But the salt-resistant activity of *VvPMA1* splicing variants was unknown. Here, the two variants were overexpressed in *Arabidopsis thaliana* and then salt tolerance of transgenic plants was measured. The results showed that the seed germination rate, root length and reproductive growth of the *VvPMA1α* and *VvPMA1β* overexpressing plants were significantly better than those of the wild type under salt stress. Furthermore, compare with *VvPMA1α* overexpressing plants, *VvPMA1β* overexpressing plants displayed superior performance. And then, *A. thaliana* with *VvPMA1β* overexpression was selected to determine its salt tolerance activity. The results showed that the growth of *VvPMA1β* transgenic plants under salt stress was better than that of wild type, and the content of proline was significantly higher than that of wild type, while MDA and Na^+/K^+ were significantly lower than that of wild type. Therefore, the *VvPMA1β* variant produced from *VvPMA1* gene alternative splicing was helpful to salt resistance of plants under salt stress.

Keywords: Grape, *VvPMA1*, overexpression, salt stress.

1. Introduction

Grape (*Vitis vinifera* L.) is a salt-sensitive plant. Under salt stress, grape roots tend to accumulate a large amount of salt, and the increase of Na^+ content in cells will produce ion toxicity, which seriously affects the normal growth and development of plants, resulting in yield loss and quality change[1]. Plants often reduce ion toxicity by Na^+ efflux, reducing Na^+ absorption, and regionalizing Na^+ into vacuoles[2]. Plasma membrane Na^+/H^+ reverse transporters can export intracellular Na^+ to reduce intracellular Na^+ accumulation, relying on the electrochemical potential gradient generated by the plasma membrane proton pump H^+-ATPase (PM H^+-ATPase) of plant cells to provide proton driving force[3]. Upregulation of intracellular proton pump can not only maintain intracellular Na^+ balance, but also is crucial for maintaining intracellular K^+ balance under salt stress[4]. Therefore, increasing PM H^+ -ATPase activity can improve the salt tolerance of plants[5].

PM H^+-ATPase is encoded by multiple genes. There are 12 genes (*AHA1-AHA12*) in *Arabidopsis thaliana*[6], There are 9 genes (*PMA1-PMA9*) in tobacco (*Nicotiana plumbaginifolia*) and 10 genes (*OsPMA1-OsPMA10*) in rice (*Oryza sativa*)[7]. The expression of different gene family members has obvious tissue specificity and environment specificity, and the biochemical characteristics of different members such as optimal pH and Km are different. For example, *AHA10* is mainly expressed in the germination seeds of *Arabidopsis thaliana*, while *AHA2* is mainly expressed in the roots[8]. Only *OsPMA7* expression in rice roots increased significantly with the decrease of environmental pH[9]. Therefore, different stress factors can affect the amount of transcription of different family members, and affect the activity of proton pump by regulating the amount of protein of certain family members.

Our previous studies have found that *VvPMA1* in the root of Crimson seedless grapes occur alternative spliced during gene expression, and splicing variant *VvPMA1β* is generated through intron retention mechanism. Compared with the constitutive isoform *VvPMA1α*, 35 amino acid residues were missing at the N-terminal of *VvPMa1β*, which just released the inhibition of the N-terminal self-inhibitory region of the plasma membrane H^+-ATPase, showing a higher plasma membrane proton pump activity in yeast[10]. However, the functions of the two variants in plant growth and

development and under salt stress have not been further studied. In this study, the *VvPMA1α* and *VvPMA1β* overexpression *A. thaliana* were acquired, and these transgenic plants growth and development was studied under salt stress .

2. Materials and Methods

2.1 Material

2.1.1 Plant Material

After being fully disinfected, the wild-type *A. thaliana* (Col-0) seeds were seeded in 1/2 MS solid medium, and placed in 4℃, 2 days for vernalization. And then transferred to a greenhouse with 22℃, 100 μmol·m^{-2}·s^{-1} and 16 h/ 8 h light period. The seedlings with uniform growth were selected and transplanted into nutrient soil for 3-4 weeks. After bolting, arabidopsis were transfected by *Agrobacterium*.

2.2 Methods

2.2.1 Construction of Overexpression Vector

The pEASY-Blunt-*VvPMA1α*/*β* plasmid preserved in our laboratory was double digested by *Bam*H I and *Eco*R I, and then the target fragment was inserted into the PRI101-AN plant expression vector, which was also double-digested by the same enzyme, and transformed into *E. coli* DH5α, and the positive clones were screened and sequenced to verification. The constructed expression vectors were named PRI-*VvPMA1α* and PRI-*VvPMA1β*. Subsequently, *Agrobacterium* with PRI-*VvPMA1α* and PRI-*VvPMA1β* were constructed for Arabidopsis transformation.

2.2.2 *A. Thaliana* Transformation

A. thaliana was transformed by flower dipping[11]. The transgenic plants were verified with the gene-specific 35S forward primer (5'-GACGCACAATCCCACTATCC-3') and the *VvPMA1α* and *VvPMA1β* reverse primer (5'- TCATACTGTGTAATGCTGTTG-3') by PCR after screening with kanamycin. The positive overexpression lines OE were randomly selected for use in subsequent experiments.

2.2.3 Determination of Physiological Indexes of Transgenic Plants under Salt Stress

The content of proline was determined by Irigoyen et al[12]. The content of malonaldehyde (MDA) was determined by Guo et al[13].The content of Na^+ and K^+ was determined by flame spectrophotometer[14].

2.2.4 Data Analysis

Excel 2019 software was used for data processing, IBM SPSS Statistics 26 statistical software for Origin2021 mapping was used for variance analysis, and T-test was used for significance analysis ($P < 0.05$).

3. Experimental Result

3.1 Identification of Transgenic A. Thaliana

VvPMA1α and *VvPMA1β*, was overexpressed in *Arabidopsis*, and T3 homozygous transgenic lines were generated. The genomic DNA of each transgenic *A. thaliana* was used as template for PCR identification using PRI-*VvPMA1α* and PRI-*VvPMA1β* specific primers, and the wild type was the negative control. The results showed that the five *VvPMA1α* (*α*OE2-6)and *VvPMA1β*(*β*OE3-7)transgenic Arabidopsis thaliana plants had corresponding target genes inserted into their genomes (Fig. 1).

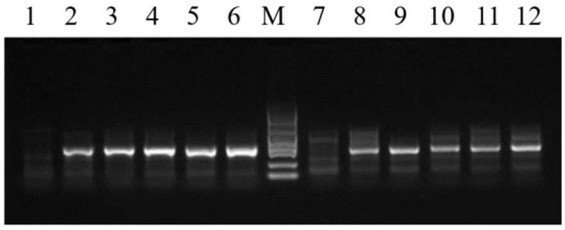

Fig.1 Identification of the transgenic A. thaliana by PCR
1, 7: Wild type *Arabidopsis*; 2~6: PRI-*VvPMA1α* transgenic *Arabidopsis* αOE2~6; 8~12: PRI-*VvPMA1β* transgenic *Arabidopsis* βOE3~7; M: DL2000 DNA Marker

3.2 The Growth of Transgenic Arabidopsis Thaliana under Salt Stress

In order to observe the growth of transgenic *Arabidopsis thaliana* under salt stress, the two transgenic plants and wild type plant were seeded in 1/2MS solid medium with 0 mmol·L^{-1} and 100 mmol·L^{-1} NaCl, and grown for 7 days (Fig 2A). Under control conditions, the growth of two transgenic and wild type plants had no difference and the germination rate of all was more than 90% after 3 days of culture (Fig 2B). Under 100 mmol·L^{-1} NaCl, the growth of both transgenic and wild *arabidopsis* were significantly inhibited. After three days of sowing, the germination rate of wild type arabidopsis was merely 36%, whereas transgenic plants exhibited a remarkable increase to over 83%. (Fig 2C). Moreover, the germination rate of *VvPMA1β* overexpressing plant was little higher than *VvPMA1α.* Therefore, compare with *VvPMA1α*, *VvPMA1β* showed a greater improvement in seed germination under salt tolerance. The subsequent experiments were carried out with *VvPMA1β*, which might have a higher activity.

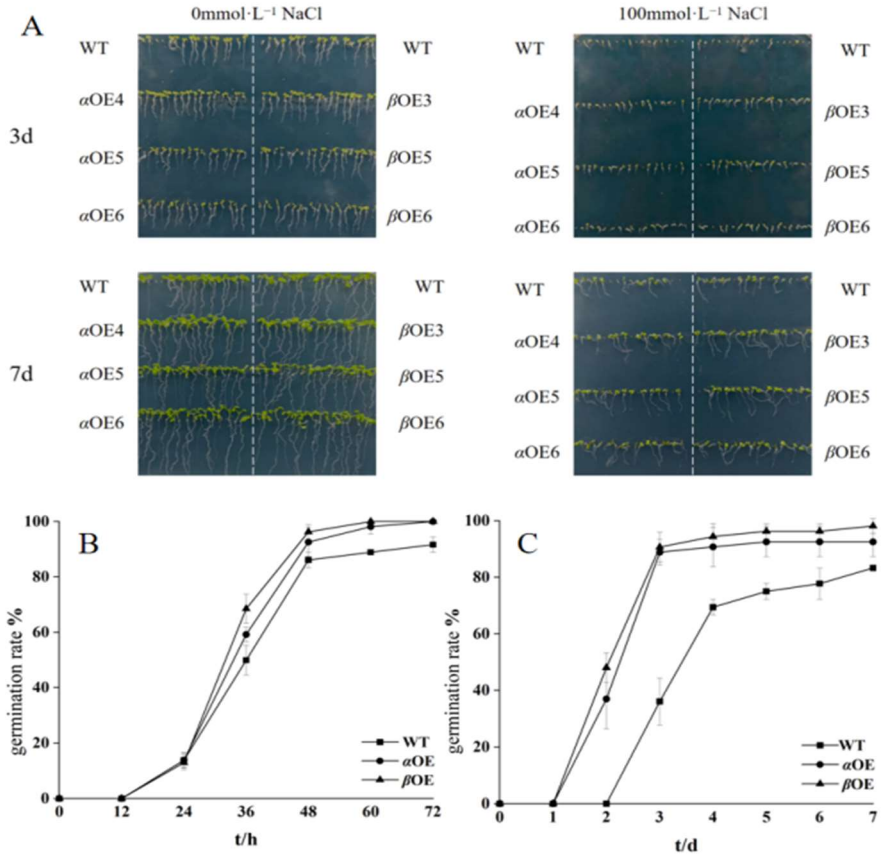

Fig. 2 Growth of transgenic *Arabidopsis thaliana* under different salt concentrations
A: Seedling phenotype of *Arabidopsis thaliana* under salt stress; B: Germination rate analysis of seeds on 1/2 MS medium (0 mmol·L^{-1} NaCl); C: Germination rate analysis of seeds on 1/2 MS medium (100 mmol·L^{-1} NaCl)

3.3 Changes of Proline and MDA Content in VvPMA1β Overexpressing Arabidopsis Lines under Salt Stress at the Seedling Stage

The proline content was not different between the WT and *β*OE lines in the control group (Fig 3A). The proline content of the WT, *β*OE3 and *β*OE5 increased by 9.87, 11.65, and 11.51% after treatment with 100 mM NaCl.

There was no significant difference in MDA content in leaves between wild type and transgenic plants under control conditions. After salt stress, MDA content increased significantly in WT leaves, but did not change in transgenic plants (Fig 3B).

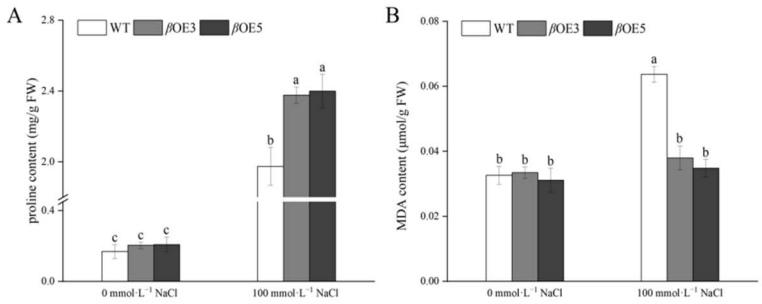

Fig. 3 Proline (A) and MDA (B) content of transgenic and wild plants after salt treatment

3.4 Changes of Na+/K+ in Arabidopsis Thaliana Leaves with VvPMA1β Overexpression under Salt Stress

Under salt stress, the accumulation of Na^+ and the loss of K^+ in plant cells caused ion damage, which seriously affected the growth and survival of plants[15]. Therefore, Na^+/K^+ has become one of the important indexes to evaluate the salt tolerance of plants. After salt stress, the contents of Na^+ and K^+ in leaves of different plants were measured, and Na^+/K^+ was calculated (Fig 4). The results showed that there was no significant difference in the content of Na^+ and K^+ in the leaves of wild type and transgenic *Arabidopsis* before salt treatment, while the content of Na^+ in the leaves was significantly lower than that of wild type, and the content of K^+ in the leaves was significantly higher than that of wild type after salt treatment. Therefore, Na^+/K^+ in the leaves of *VvPMA1β* transgenic *Arabidopsis* was significantly lower than that in wild type plants, indicating that overexpression of *VvPMA1β* reduced the accumulation of Na^+ and increased the accumulation of K^+ in the leaves of *Arabidopsis thaliana*, ensuring lower Na^+/K^+ in the leaves, which was conducive to the growth of plants under salt stress.

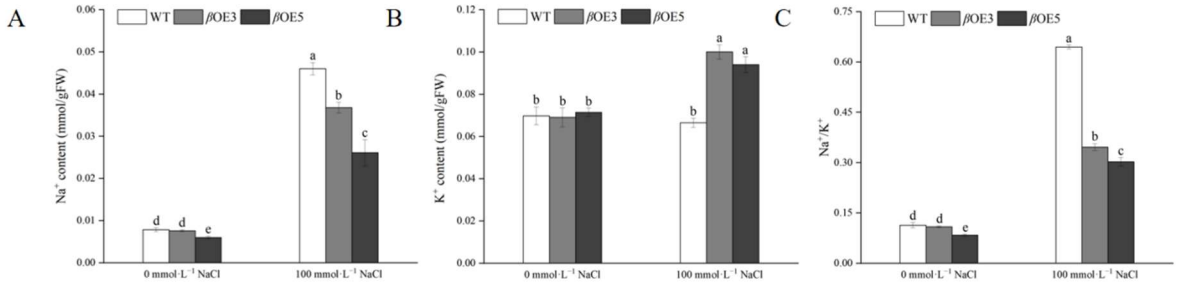

Fig. 4 Na^+ (A), K^+ (B) content and Na^+/K^+ (C) of transgenic and wild plants after salt treatment

4. Discussion

A large number of studies have reported that plasma membrane H^+-ATPase can participate in plant salt resistance. For example, Wang et al.[16]found that *PeHA1* gene of *populus eueutica* significantly enhanced the homeostasis control ability of ions and reactive oxygen species in salinized plants. In addition, the expression and activity of H^+-ATPase in the plasma membrane of Kallar grass increased

with the increase of salt stress[17]. However, H^+-ATPase belongs to a multi-gene family, and different H^+-ATPase family members have obvious tissue specificity and environmental specificity[18].

VvPMA1 alternative splice occurred in grape root under salt stress, forming a splicing variant *VvPMA1β* with 35 amino acid residues missing at the N-terminal. Moreover, it was found the H^+ transport capacity of *VvPMA1β* is higher than that of the constitutive spliceosome *VvPMA1α*[10] in the functional complementation test of the H^+-ATPase deletion mutant RS-72. So, we supposed that the increased expression of *VvPMA1β* may be more beneficial to the salt tolerance of plants. To verify this hypothesis, two variants of *VvPMA1α* and *VvPMA1β* were transformed into *Arabidopsis.* And the results displayed that overexpression of *VvPMA1α* and *VvPMA1β* in Arabidopsis enhanced the germination rate and promoted root growth. Moreover, *VvPMA1β* overexpression was more effective compare to *VvPMA1α* overexpression in terms of salt tolerance.

Plants under stress adapt to the environment by accumulating compatible osmolytes[19]. Proline acts as important osmolyte that accumulates in plants under stress conditions[20]. Proline content increased under salt stress in all lines and that the highest is in the *β*OE lines (Figure 3A). These results showed that overexpressing *VvPMA1β* may increase the osmotic adjustment rate in Plants.

NaCl stress leads to the accumulation of MDA, an end-product of membrane peroxidation[21]. Therefore, lower MDA content may reflect stronger resistance to salt stress[22]. In the present study, MDA content increased under salt stress. The MDA content was lower in the *β*OE lines. It suggested that *VvPMA1β* transgenic plants could improve the salt-resistant ability of plants by reducing membrane oxidation damage.

Furtherly, overexpression of *VvPMA1β* reduced the accumulation of Na^+ in leaves and maintained the intracellular Na^+/K^+ at a low level (Fig 4A,C). Because *VvPMA1β* overexpressing can upregulate the proton pump activity, more H^+ is transported from intracellular to extracellular space, thereby improving the transmembrane electrochemical potential gradient. This increase in gradient provides more energy for the plasma membrane Na^+/H^+ antiporter to decrease cytoplasm Na^+ accumulation , resulting in an increase in plant salt tolerance [16, 23].

5. Conclusion

In summary, overexpression of *VvPMA1β* increased the tolerance to salt stress in Arabidopsis during the germination and seedling stages. The improved salt tolerance was the result of the combined contributions of multiple mechanisms, including the regulation of ion transporters and accumulation of osmoregulating substances, and maintenance of membrane stability. This study provides a theoretical basis to study resistance to stress in plants.

Acknowledgments

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