Ectopic expression of VyCIPK1 gene, isolated from wild grape Vitis yanshanesis J, X. Chen., confers the tolerance to salt in transgenic tobacco

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ABSTRACT
Calmodulin-like interacting protein kinases play an important role in plant response to abiotic stresses and development. But the role of the CIPK gene in grapevine is unknown. In this study, VyCIPK1, isolated from the Chinese wild grape V. yanshanesis, was strongly induced by salt stress. Overexpressing VyCIPK1 could induce AOC and AOS, and result in notably increased jasmonate levels in tobacco. Under salt stress, transgenic plants showed higher germination rate, leaf number, and fresh weight than wild-type plants. Moreover, transgenic plants displayed higher chlorophyll content, catalase activity, peroxidase activity, superoxide dismutase activity, and lower malondialdehyde content, H$_2$O$_2$, and O$_2^-$ content than that of wild type under salt stress conditions. And the stress-related genes, including ERD10C, ERD10D, LEA5, POD, SOD, and CAT, were up-regulated in transgenic plants. Our founding demonstrated that the VyCIPK1 has the potential for grape molecular breeding of salt tolerance as a candidate gene.

Introduction
Soil salinity impacted over 800 million hectares of the global area and arrested plant growth and yield which are sessile organisms and evolve elaborate adaptations for reducing the effect of salt stress (Munns and Gilliham 2015; Yang and Guo 2018). Calcium is a ubiquitous second messenger that senses and transmits external stimuli into cellular to play a key role in plant response to salinity and other abiotic stresses (Xi et al. 2017). Ca$^{2+}$ was firstly perceived by Ca$^{2+}$-sensing proteins, including calmodulins (CaMs) (Kudla et al. 2010), calcium-dependent protein kinases (CDPKs) (White and Broadley), and the plant-specific calcineurin B-like proteins (CBLS) with four EF-hand sites to capture the calcium signals at N-terminus and a PPFP motif at C-terminal half for specific phosphorylation with CBL interaction protein kinases (CIPKs) which are also referred to as sucrose non-fermenting 1 related kinase 3 (SnRK3) (Xiang et al. 2007). CIPKs consist of the C-terminal regulator NAF/FISL domain required for binding with CBLS (Guo et al. 2001) and N-terminal serine/threonine protein kinase domain phosphorylated downstream proteins to transduce the perceived Ca$^{2+}$ to the consequent response (Albrecht et al. 2001).

Recent studies revealed 26 CIPKs from Arabidopsis (Kudla et al. 1999; Liu et al. 2019), 33 CIPKs from Oryza sativa (Giong et al. 2015), and different numbers throughout the plant kingdom (Zhang et al. 2019; Wang and Li 2022). The CIPK proteins have been revealed to play an important role in plant response and adaption to abiotic stresses, such as high salt, cold, drought, high pH (Huang et al. 2019; Kete- houli et al. 2021; Li et al. 2022; Zhu et al. 2022), and different developmental processes (Luan et al. 2009; Weinl and Kudla 2009). In Arabidopsis, the salt overly sensitive (SOS) pathway regulated the Na$^+$ exclusion process to maintain ion homeostasis under salt stress. As a CIPK protein, SOS3 can perceive Ca$^{2+}$ signal triggered by salinity, and phosphorylate the downstream target SOS1 gene which maintains a lower Na$^+$ elevation in the cytosol (Zhu 2003; Xu et al. 2006). And the change of numerous CIPK expressions is positively correlated with the resistance to salt in Arabidopsis (Kim et al. 2003; Kim et al. 2007; Nath et al. 2019). In Oryza sativa, the OsCIPK15 gene was induced by PEG, drought, and salt stress, and overexpressed OsCIPK15 transgenic rice enhanced the resistance to high salt (Xiang et al. 2007). Besides, overexpressed CdtCIPK5 of tripleoid bermudagrass in rice leads to increased resistance to salt and lower Na$^+$ accumulation based on detecting the relative water content, chlorophyll content, $F_v/F_m$, and transcript levels of OsNHX3, OsHKT1;1, OsHKT1;4, and OsHKT1;5 (Huang et al. 2020). Moreover, in Upland cotton, the salt tolerance of transgenic cotton was improved by scavenging ROS and involving in MAPK signaling pathways of overexpressing GhCIPK6a (Su et al. 2020). Recently, GmPKS, a CIPK from soybean, was induced by salt-alkali or other abiotic stress and located in the nucleus and cytoplasm. The proline content, antioxidant enzyme activities and stress-related gene expression of overexpressed GmPKS in Arabidopsis, and soybean hairy roots were increased which lead to improved salt resistance (Ketehouli et al. 2021). It was revealed that there are 20 CIPKs in Vitis vinifera (Xi et al. 2017), and several CIPKs from grape were characterized. VvCIPK3 and VvCIPK4 could induce the inward K$^+$ channel VvK1.2 in ripening berries (Cuéllar et al. 2010). For instance,
Arabidopsis constitutively expressed VaCIPK02 that was sensitive to salinity but more resistant to drought stress (Xu et al. 2020). However, the roles of plenty CIPKs in the regulation of plant resistance to salinity in grapes remain unclear.

Grapevine (V. vinifera L.) is an economically important fruit species that is mainly grown in soil with a salinity of 0.14% to 0.24%. High salt will hinder organ development and result in the abscission of leaves and fruits which influence the reduction of yield and quality of grapevine (Ismail et al. 2012). By contrast, China is one of the important origins of Vitis where some resistant germplasm resources are found, such as Vitis amurensis and Vitis yanshanesis (Xu et al. 2014; Xia et al. 2017; Cui et al. 2020). Xia et al. found that V. yanshanesis is highly salt-resistant and could survive in an environment with 0.6% NaCl (Xia et al. 2017). To better understand the salt tolerance mechanism, we investigated the molecules’ basis of salt stress adaption of V. yanshanesis by transcriptome analysis (unpublished). In this research, a salt-induced gene VyCIPK1, isolated from transcriptome analysis, was identified and characterized to expand the understanding the function of CIPK in grapevine.

Results

Physiochemical changes of V. yanshanesis in response to high salt

To better understand the salt tolerance, two-year-old V. yanshanesis and rootstock 5BB seedlings were conducted with 0.1 M NaCl in the greenhouse. 14 days post-treatment, the edge of the detached leaves of the 5BB exhibited the visible yellowing phenotype compared with the leaves in a similar-position of V. yanshanesis. 5BB leaves retained 61% of the chlorophyll concentration, while V. yanshanesis retained 80% of the chlorophyll concentration. After 21 days, some of the etiolated leaves of 5BB had dried and fallen over, whereas there was only a yellow phenomenon in the counterpart of V. yanshanesis. Further physiological analysis revealed that the basal leaves of V. yanshanesis still had higher chlorophyll content of 24.7% than those 5BB which only had 4.3%. These results suggest that V. yanshanesis is more tolerant of salt stress (Figure 1).

Candidate genes involved in salt stress

The CIPKs serve as the main downstream Ca2+ signaling components involved in salt stress (Luan et al. 2009). Due to the DEGs analysis of V. yanshanesis under salt stress, 9 CIPKs were identified to express differentially including 3 up-regulated CIPKs and 6 down-regulated CIPKs (Table 1). The CIPK expression patterns in DEGs analysis were consistent with the results carried out by qPCR (Figure 2). Among the 9 CIPKs, the most up-regulated CIPK gene, VIT_08s0058g1040, which was high homologous to VvCIPK07 gene (GenBank No. NP_001268154), was isolated and identified to be further studied to elucidate its mechanism of salt resistance (Figure 2).

Figure 1. Phenotypic and physiochemical changes of Vitis yanshanesis and 5BB plantlets treated with 0.1 M NaCl. (A) The phenotypic change of V. yanshanesis and 5BB plantlets treated with NaCl for 21 days. (B) The phenotypic change of V. yanshanesis and 5BB leaf post-treatment with NaCl at 0, 14, and 21 days. (C) The chlorophyll concentration change of V. yanshanesis and 5BB leaf was affected by NaCl treatment at 0, 14, and 21 days.
Based on the DEG sequence and the corresponding VIT_08s0058g1040 gene, we designed the primers and cloned the full-length sequence of the CIPK gene, named VyCIPK1. The coding sequence is 1380 bp, which encodes a putative protein of 460 amino acids with a predicted protein molecular weight of 51.5 Kd (GenBank No. MK942081). Chromosome location analysis showed that VyCIPK1 is mapped on chromosome 8, including 1 intron (9 bp) and 2 exons (1323 and 57 bp) (Figure 3(A)). Amino acid sequence analysis indicated that VyCIPK1 has a high similarity 98% with VvCIPK07 (GenBank No. NP_001268154), but there exists 7 amino acid mutant sites (Position: 13, 61, 67, 274, 283, 311, and 338) and 1 deletion of the amino acid fragment (Position: 443) suggesting VyCIPK1 is a new member of CIPK gene family. The alignment of the VyCIPK1 protein sequence with 3 CIPK proteins from other species reveals that VyCIPK1 protein contains a conservative serine/threonine protein kinase domain S_TKc at N-terminal (Position: 13-268) and a regulatory domain NAF at C-terminal (Position: 314–363)(Figure 3(B)). A Sequence alignment analysis of CIPK proteins showed that VyCIPK1 also has 70–90% identities with JrCIPK2 (Julans regla) and OsCIPK15 (Oryza sativa). A phylogenetic analysis of 34 plant CIPK proteins showed that VyCIPK1 belonged to the same cluster as AtSOS3, AtCIPK2, OsCIPK15, AtCIPK15, OsCIPK10, and AtCIPK20 (Figure 3(C)). In Arabidopsis, SOS3 enhanced salt tolerance by regulating the intracellular and extracellular sodium and potassium balance (Xu et al. 2006). Overexpression of OsCIPK15 in Oryza sativa increased the tolerance to abiotic stresses, such as drought, cold, and high salt (Xiang et al. 2007). Therefore, we predicted that VyCIPK1 was involved in salt stress.

Localization of the VyCIPK1 to cytomembrane and cytoplasm

To reveal the cellular basis of a gene, subcellular localization of the VyCIPK1 was tested via transient genetic transformation in Arabidopsis mesophyll protoplasts. We created a construct harboring a VyCIPK1-GFP fusion gene, in which GFP was fused to the 3′ end of the VyCIPK1 gene. Figure 4 shows that VyCIPK1 fused protein was exclusively localized in the cytomembrane and cytoplasm, whereas the control GFP protein was distributed throughout the cell, including the nucleus, cytoplasm, and cytomembrane (Figure 4). The results indicated that VyCIPK1 localized in the cytomembrane and cytoplasm.

Overexpression of VyCIPK1 in tobacco results in dwarfing

Since VyCIPK1 was induced by salt, it was inferred that this gene could function in salt stress. To investigate this, VyCIPK1 was overexpressed in Nicotiana benthamiana. Two independent transgenic lines, OE1 and OE3, with equivalent levels of VyCIPK1 transcript were chosen for further study. The 30-day-old transgenic tobacco plants showed a significant dwarfed phenomenon that the size was prominently smaller than the corresponding wild type. The heights of transgenic plant lines OE1 and OE3 were 24.8% and 24.6% of wild type, respectively. And the leaf areas of transgenic plant lines OE1 and OE3 were only 82.4% and 78.9% of the control, respectively (Figure 5). The results suggested that overexpression of VyCIPK1 could impair the developmental processes of transgenic plants.

Analysis of phytohormone levels of transgenic tobacco

To illustrate the mechanism of the dwarf phenomenon of transgenic tobacco, the phytohormone was detected by the

| ID number      | Log2(FC) 6 h vs 0 h | Log2(FC) 12 h vs 0 h | Gene function                                              |
|----------------|---------------------|----------------------|------------------------------------------------------------|
| VIT_09s0167g00160 | −2.20936            | −3.50619             | CBL-interacting protein kinase 02                          |
| VIT_11s0061g00200 | −1.21896            | −1.30143             | CBL-interacting protein kinase 06                          |
| VIT_08s0058g01040 | 1.05064             | 2.742074             | CBL-interacting protein kinase 07                          |
| VIT_04s0044g00630 | 0.82539             | 1.06025              | CBL-interacting protein kinase 08                          |
| VIT_06s0004g07870 | −2.06191            | −1.74956             | CBL-interacting protein kinase 10                          |
| VIT_06s0004g07830 | 1.467608            | 1.64372              | CBL-interacting protein kinase 11                          |
| VIT_13s0067g02480 | −1.21842            | −1.45094             | CBL-interacting protein kinase 13                          |
| VIT_11s0052g01700 | −2.17591            | −3.04077             | CBL-interacting protein kinase 14                          |
| VIT_10s0003g01410 | −3.53735            | −6.90756             | CBL-interacting protein kinase 16                          |

Figure 2. CBL-interacting protein kinase gene expression patterns involved by salt stress were validated by qRT-PCR to RNA-seq.
Figure 3. (A) Chromosome location and schematic representation of VyCIPK1 gDNA. (B) Sequence alignment analysis of VyCIPK1 with VvCIPK7 (V. vinifera), JrCIPK2 (Julians regla), and OsCIPK15 (Oryza sativa). Accession numbers are JrCIPK2 (XP_018819314), OsCIPK15 (AK121773), and VvCIPK7 (NP_001268154). (C) Phylogenetic tree of CIPK proteins from grapevine and other species using the neighbor-joining method by the MEGA 5.0 software.
LC-MS/MS method. There was no big difference in auxin, cytokinins, gibberellin acids, and SA levels between WT and transgenic tobacco. By contrast, the jasmonates, containing JA, H2JA, and JA-ILE in transgenic tobacco, were all significantly higher than that of WT (Figure 6(A)). Meanwhile, the AOS and AOC, the genes encoding key enzymes involved in jasmonic acid biosynthesis, were detected by qPCR. It was shown that the AOS and AOC gene were markedly elevated by overexpressing VyCIPK1 in tobacco, which coincided with the JA content results (Figure 6(B)).

**Salt tolerance analysis of transgenic tobacco plants**

To confirm whether VyCIPK1 affects salt resistance during germination or seedling growth stages, tobacco seeds transgenic and WT were sterilized and kept on the 1/2 MS medium with or without NaCl. Wild-type and OE lines showed little difference in germination rate on MS medium. However, seeds of OE lines could have 82.5% (OE1) or 72.5% (OE3) germinated, which were significantly higher than that of wild type (37.5%) within 8 d under 0.1 M NaCl. While in the presence of 0.15 M NaCl for 8 d, seed germination of OE lines was still obviously higher with 62.5% (OE1) and 55% (OE2) than that of WT (15%) (Figure 7).

While treated with 0.1 M NaCl for 12 d, the leaf number of OE lines was about 3.81 (OE1) or 3.45 (OE3), compared with 2.36 of wild type. When treated with 0.15 M NaCl for 12 d, the leaf number in transgenic tobacco was about 3.55 (OE1 line) or 3.45 (OE3 line), which was slightly higher than that of WT (2.18). In contrast, the fresh weight was...
significantly greater in the transgenic lines, 2–3 folds as that of wild-type plants by 0.1 M or 0.15 M NaCl treatment (Figure 8). This result exhibited that overexpression of VyCIPK1 could increase the salt resistance of tobacco at germination and vegetative stage.

Six-week-old tobacco plants were exposed to salinity (0.1 M NaCl) for 2 weeks. The leaves of transgenic tobacco lines exhibited to be more thickened and greener with the higher chlorophyll content (25–26 mg/g) and the higher potential quantum efficiency of photosystem II than that of wild-type tobaccos which retained 60% of their initial Chlorophyll content (10.13 mg/g) (Figure 9(B)).

The cell permeability-associated parameters ion leakage level and membrane lipid peroxidation relative indicator, the content of malondialdehyde (MDA) and H$_2$O$_2$, were measured to represent the physiological status of the tobacco plants’ response to salt stress. There was no obvious difference in ion leakage level between transgenic and wild-type tobacco in the absence of salt. When treated with 0.1 M NaCl, WT showed greater sensitivity to salt in the change of ion leakage level than transgenic lines: salinity led to a significant increase in WT, which was 1.42–1.43 times as that of transgenic plants, respectively (Figure 10(A)).

There was no significant difference in MDA and H$_2$O$_2$ content in transgenic and wild-type tobacco under normal conditions. The MDA and H$_2$O$_2$ content induced by salinity were significantly higher in WT (MDA: 12.88 µmol g$^{-1}$; H$_2$O$_2$: 8.74 µmol g$^{-1}$), which was 1.74–2.14-fold or 1.63–1.83-fold times as that of transgenic plants (Figure 10(B, C)). The results indicated that the level of membrane lipid peroxidation confront salt was lower when overexpressed VyCIPK1.

To confirm that, the antioxidant enzymes CAT, POD, and SOD were measured with or without salinity. Figure 10 shows that CAT, POD, and SOD activities significantly increased post-salt treatment, and the activities of CAT, POD, and SOD in transgenic plants rose greater than that in WT (Figure 10(D–F)). Thus, it was inferred that ROS
induced by salt stress that resulted in cellular membrane lipid peroxidation and was harmful to the cell permeability could be decreased by improving the several antioxidant enzymes and reducing the reactive oxygen species, such as H₂O₂ via overexpressed the VyCIPK1 gene, the salt tolerance to salinity of transgenic plants was thereby enhanced.

VyCIPK1 regulated the expression of stress-responsive genes under salt stress conditions

To further reveal the molecular mechanism of VyCIPK1, the related genes, including ROS-scavenging enzymes (CT1, SOD, and POD), probiosynthetic gene (P5CS and P5CR), and early responsive drought genes (ERD10C, ERD10D, and DREB) were analyzed via qPCR (Figure 11). Under normal condition, the expression levels of antioxidant enzymes NtACT1, NtSOD, and NtPOD were higher in transgenic plants which were consistent with the previous result that the content of ROS-scavenging enzymes was lower in transgenic tobaccos without NaCl. Meanwhile, the expression levels of P5CS1 and P5CR were lower in transgenic plants which validated that overexpressing VyCIPK1 could inhibit vegetative growth and result in the dwarf phenomenon. ERD10C and ERD10D were higher in transgenic plants. When subjected to salt stress, the expression of all genes increased compared to normal conditions; however, the increased expression levels in transgenic plants were greater than in wild-type plants and contributed to the higher salt tolerance of the VyCIPK1-overexpressing seedlings. Although the DREB3 gene was lower expressed in transgenic lines, the increment of this gene was still much higher in the tobacco-overexpressed VyCIPK1. Accordingly, VyCIPK1 could modulate the expression of stress-responsive genes to enhance salt resistance.

Discussion

Salt stress formed the hyperosmotic condition and triggered ionic stress, osmotic stress, and secondary stress, particularly oxidative stress in plants and which resulted in photosynthesis reduction, developmental changes, growth, and yield inhibition (Yang and Guo 2018). Ca²⁺ works as an essential signal agent and could sense, deliver, and respond to abiotic stress (Zhu 2003). The CIPKs serve as the main downstream Ca²⁺ signaling components involved in salt stress. In this study, there were 9 CIPK genes known to relate to abiotic stresses, which were detected to express differentially (Table 1). Among the 3 up-regulation CIPK genes, VyCIPK1, which was the most up-regulated post-salt stress was isolated and characterized (Figure 2). Besides, VyCIPK1 that had 2 exons located on chromosome 8 was homologous to VvCIPK7 from V. vitis, OsCIPK15 from Oryza sativa and JrCIPK2 from Juglans regla (Figure 3). The VyCIPK1 gene was induced by high salt stress which was consistent with the expression pattern of OsCIPK15 which could positively

Figure 7. Seed germination of WT and transgenic lines overexpressing VyCIPK1. (A) Seed germination of WT, transgenic lines OE1 and OE3 under 0.1 M NaCl or 0.15 M NaCl for 6 d (i), 8d (ii), and 12 d (iii). (B) Phenotypes of WT, OE1, and OE3 grew in normal conditions. (C) Germination rate of WT, OE1, and OE3 under normal conditions or different salt stresses (0.1 M NaCl or 0.15 M NaCl) for 8 d. The data represent means ± SD of three replicates and the statistical analyses were performed with a one-way ANOVA test, *p < 0.05.
regulate the stress sensor SnRK1A to regular the sugar and energy production and promote the elongation of coleoptile (Lee et al. 2009; Tsai and Gazzarrini 2012; Simon et al. 2018; Ye et al. 2018).

It was shown that transgenic rice constitutively expressing the homolog gene OsCIPK15 showed enhanced resistance to salt stress (Xiang et al. 2007). To detect whether VyCIPK1 affects salt tolerance, VyCIPK1 was overexpressed in tobacco and the response to salinity was investigated. Consistently, the transgenic tobacco resulted in enhanced salt tolerance, based on diverse aspects such as the development process (seed germination, leaf number, fresh weight) (Figures 7 and 8), photosynthesis (FV/FM, PSII, chlorophyll) (Figure 10), cell permeability (ion leakage level), and membrane lipid peroxidation (MDA and H2O2) when subjected to salinity (Figure 10). Meanwhile, the content of peroxidase (POD, CAT, and SOD) and the expression of the assistant ROS scavenging enzyme genes were higher in the VyCIPK1 overexpressed tobacco relative to the wild type (Figures 10 and 11).

ROS is rapidly induced and accumulated by salt stress that not only plays an important role in signaling molecules in response to abiotic stress but also has oxidative stress-induced toxic effects. Thus, it is important to regulate ROS homeostasis at suitable levels under salt stress (Mittler et al. 2004; Miller et al. 2008). And numerous enzymatic scavengers are involved in the alleviation of ROS-induced damage in plants in salinity (Xi et al. 2017). The CBL1/9-CIPK26 could phosphorylate AtRbohF, a plant ROS-producing NADPH oxidase that forms ROS under salt stress, which indicated that the CBL-CIPK signal pathway interacted with the ROS production network (Drerup et al. 2013). In addition, the constative expression of PpSnRK1α, the homolog interacting gene of OsCIPK15, had higher expression of the peroxidase gene and enhanced ROS scavenging capacity under salt stress (Wang et al. 2020). Similarly, overexpressing VyCIPK1 could interact with the ROS network and decrease the damage to the plant cell, which could improve the salt tolerance in transgenic plants.

Plants have evolved complex mechanisms for adapting to salt stress including ROS, calcium ions, and JA (Chen et al. 2017). Calcium-dependent protein kinases of Nicotiana attenuate silenced in transgenic plants showed remarkable increased JA concentrations and stunted stem elongation (Heinrich et al. 2013), which showed that the Ca2+ pathway could interact with the JA signal pathway and regulate the plant growth and the resistance to salt stress. In this study, it was found that AOC and AOS, key enzymes involved in JA biosynthesis were elevated in response to overexpressing the VyCIPK1 in tobacco, which resulted in elevated levels of JAs (Figure 6). JA plays a key role in plant development and environmentally stressed including salt stress (Ma et al. 2006; Ismail et al. 2012; Lian et al. 2013). For instance, the RICE SALT SENSITIVE3 gene adapted to salinity by regulating the JA-responsive genes. Besides, JA-ILE and allene oxide cyclase-derived jasmonates could inhibit plant growth by regulating gibberellin metabolism (Yang et al. 2020). It is inferred that the VyCIPK1 gene could induce the JA biosynthesis to make a contribution to adapting salinity and growth impeded. The dwarfing phenomenon overexpressed VyCIPK1 coincided with that overexpression of salt-tolerant related genes may regulate the osmotic adjustment to adapt.
high saline conditions hampered the growth rate by redirecting energy sources to the formation of organic solutes as well (Munns and Gilliham 2015).

Except for JA, salt-induced growth changes via other hormone levels, particularly ABA, which has a major role in growth and saline adaption (Geng et al. 2013). It was illustrated that VaCIPK02 of Amur grape interacts with PYL9, an ABA receptor to regulate the ABA-responsive genes and modulate ROS levels, negatively regulates the salt tolerance in Arabidopsis (Ismail et al. 2012). In our experiment, the
transcript level of DREB, the dehydration-responsive element-binding protein that was a functional protein resistant to salt stress involved in the ABA signal pathway (Lata and Prasad 2011), was detected in transgenic tobacco and was slightly higher in WT without NaCl, but significantly induced by salt and more highly expressed in the overexpressing VyCIPK1 lines (Figure 11). It was indicated that VyCIPK1 may also have affected the ABA signal pathway to regulate the salt resistance which needed to be investigated further.

P5CR and P5CS catalyze glutamate synthesis into proline which is a key osmolyte to maintain osmotic pressure, cell volume, and turgor in plants upon salt treatment (Armen-gaud et al. 2004; Guan et al. 2018). It was reported that overexpression of P5CS had significantly higher proline accumulation and enhanced salt, osmotic, and drought tolerance (Wei et al. 2016). Consistently, higher transcript levels of P5CR and P5CS in transgenic tobacco of VyCIPK1 were detected when subjected to salt stress (Figure 11). For instance, higher expression levels of ERD10C and ERD10D that are rapidly induced by salt-induced dehydration and function as a chaperone for protecting proteins against abiotic stress (Kovacs et al. 2008) were observed in VyCIPK1 transgenic tobaccos than wild types.

In conclusion, a salt-induced VyCIPK1 gene was isolated from the Chinese wild grape V. yanshanesis. Overexpression of VyCIPK1 in tobacco enhanced the tolerance to salt stress via ROS-scavenging enzymes, membrane systems, and stress-responsive genes. In addition, VyCIPK1 increased the jasmonate levels in tobacco by the regulation of the key genes of jasmonic acid biosynthesis AOC and AOS. These findings suggested that the VyCIPK1 gene may enhance salt tolerance by regulating the JA signal pathway, which was helpful to understand the salt resistance mechanism of the Chinese wild grape.

Methods

Plant material treatment

Two-year-old potted seedlings of V. yanshanesis and 5BB with pots of 40 cm diameter and 30 cm height were maintained in the Grape Repository of Shandong Institute of Pomology. Firstly, the salt treatment was conducted by watering with 0.1 mol/L NaCl to soil saturation till flowing from the bottom of the pot and then watered with 3 L NaCl solution every 7 days. The leaf samples were harvested at 0, 6, 12 h, 14, and 21 d after treatment and immediately frozen in liquid nitrogen. The samplings for each treatment were collected from three independent seedlings which were of consistent growth status.

N. benthamiana was used for genetic transformation which was for further detecting the change of seed germination and salt resistance. For the seed germination experiment, the T2 transgenic tobacco seeds were immersed in 10% (v/v) sodium hypochlorite for 20 min, followed by rinsing with sterile distilled water three times. Then the sterilized seeds were cultured on a solid MS medium containing 0.1 M or 0.15 M NaCl at 4°C for 2d to break
the dormancy, and by maintaining at 25–28°C and 2000 lx under a 14 h photoperiod regime. The germination rate was continuously collected. And for salt treatment, four-week-old tobacco plants were watered with 0.1 mol/L NaCl until the solution oozed. After 10 d, the tobacco growth and salt physiological indices were measured.

**Gene cloning**

VyCIPK1 forward primer (5'-ATGGCGTCACGGTCAAG-TAC-3') and reverse primer (5'-CTAACATTATTCACCTTCTCCTTTTAT-3') were designed according to the homologous sequences VyCIPK1 gene retrieved from the V. vinifera cultivar Pinoir Noir 40024 (https://www.genoscope.cns.fr/externe/Genome-Browser/Vitis/).

Total RNA was isolated from V. yanshanesis leaves by Omega RNA Kit R6827. The first-stand cDNA was obtained using a cDNA Synthesis kit (PrimerScript™ II 1st Strand DNA Synthesis Kit, Takara). VyCIPK1 gene was amplified from the cDNA of V. yanshanesis leaves by RCR reactions as follows: 95°C for 2 min followed by 30 cycles (98°C, 10s; 60°C, 10s; 72°C, 2 min), then 72°C, 2 min. Reactions were performed using 5 μL 5 × PrimerSTAR (Mg2+ Plus) buffer, 2 μL dNTP Mixture, 0.2 μL each primer (10 μmol/L), 0.2 μg cDNA template, 0.25 μL PrimerSTAR DNA Polymerase, adding ddH2O to 25 μL. PCR products were extracted by the Gel extraction kit (OMEGA, USA) and sequenced by Sangon Biological Engineering Technology in Shanghai.

**Sequence analysis of VyCIPK1 and its encoded protein**

Chromosomal location of VyCIPK was predicted using the Grape Genome Browser (12X)(https://www.genoscope.cns.fr/externe/Genome-Browser/Vitis/). Protein molecular weight and isoelectric point of VyCIPK were identified by ProP (https://web.expasy.org/protparam/). And the conserved domain and function were predicted by NCBI blastX (https://www.ncbi.nlm.nih.gov//Blast. cgi). A multiple alignment analysis was conducted by DNAMAN. The phylogenetic dendrogram was constructed by MEGA 5.0 software according to the Neighbor-Joining method.

**Gene expression pattern analysis and subcellular location**

VyCIPK expression was carried out by qRT-PCR with a Bio-Rad CFX Real-time PCR Detection System utilizing SYBR premix Ex TaqII Kit TaKaRa. qRT-PCR reactions were carried out using 10 μL SYBR premix Ex Taq II, 0.8 μL each primer, and 2 μL DNA template, adding ddH2O to 20 μL. The qRT-PCR reaction was conducted in triplicate as follows: 95°C for the 30s followed by 40 cycles (98°C, 5s; 60°C, 10s; 72°C, 30s). The normalized fold expression of RNA was counted by comparison with the control of the VvActin gene via the 2^-ΔΔCT method (Livak and Schmittgen 2001). The quantitative PCR primers were designed by Primer Primer 5.0 (Table S1).

To test the VyCIPK1 subcellular localization, the VyCIPK1 coding region was fused to GFP in the pBI221-GFP plasmid to construct the VyCIPK1-GFP vector. For subcellular localization assays, Arabidopsis protoplasts were isolated according to a previous protocol (Yoo et al. 2007). The vector was transferred to the protoplasts using the PEG6000 method. The transfected protoplasts were incubated for 12–16 h at 28°C in the dark. Fluorescence was detected using a confocal laser scanning microscope (LSM510; Carl Zeiss Thornwood, New York, NY, USA).

**Plant transformation**

The coding sequence of VyCIPK1 was amplified with the primer 5'-gGCATGatatagaaattgagatc 3' and 5'-gGGTAACCTtaggaagtgagatc 3'. The generated fragments were digested by restriction endonucleases Bgl II and BstE II, then inserted into the vector pCAMBIA1301 replacing the GUS gene to produce the construct pCAMBIA1301::VyCIPK1. These constructs were transformed into N. benthamiana using the Agrobacterium GV3101 by the leaf disk method according to the protocol described by Horsch et al. (1985). The screening of transgenic tobacco plants was conducted on MS medium hygromycin (25 mg L^-1) added. The T2 transgenic tobacco plants were grown in a greenhouse.

**Physiological responses of transgenic tobacco under salt stress**

For the chlorophyll content test, 0.1 g cutting leave sample in 10 mL extracting solution (aceton: ethanol = 1:1, v/v) was kept in dark at a growth incubator until mesophyll tissue turn white, followed by measured by UV spectrophotometer at 645 and 663 nm. The chlorophyll content =\((20.2 \times OD_{645nm} + 8.02 \times OD_{663nm}) \times V/1000/W\), V, volume of extraction solution (mL), W, fresh weight of leaf sample (g).

Quantum efficiency (Fv/Fm) and the relative electrolytic leakage were investigated as previously described by Huang et al. (2020) and Xu et al. (2014), 0.3 g fresh leaf sample washed with deionized water was chopped and immersed in 10 mL deionized water for 3 h to conduct the solution electrolytic leakage R1. After boiling for 20 min and cooling to room temperature, the solution electrolytic leakage R2 was tested. Relative electrolytic leakage (%) = R1/R2 × 100%.

To investigate the antioxidant enzyme activity, the SOD, POD, and CAT activities were tested respectively according to Liang et al. (2003). SOD activity was conducted by the nitroblue tetrazolium (NBT) Illumination method, and 1U SOD activity was equivalent to the enzyme that inhibited 50% NBT illumination. POD activity was tested by the Guajacol method, and 1U equals the enzyme that decreases OD_{470} value by 0.01 per minute. CAT activity was measured by ultraviolet spectroscopy, and 1 U equals the enzyme that decreases OD_{240} value by 0.01 per minute. All the enzyme activity is indicated as U mg^-1 protein.

**Analysis of phytohormones**

For the phytohormone content test, a 50 mg leaf sample pulverizing with a grind machine (MM 400, Retsch) at 30 Hz for 1 min was dissolved in extracting solution (methyl alcohol: water: formic acid = 15: 4: 1, v:v:v), followed by solution concentrated. The concentrated liquor was redissolved in 100 μL 80% methanol and filter-sterilized with a 0.22 μM PTFE filter membrane. Phytohormone contents were detected by the LC-MS/MS analysis according to MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP
6500 LC-MS/MS platform. Each assay was performed in triplicate.

**Statistical analysis**

In this study, each experiment contained three biological replicates. A statistical analysis was performed with a one-way ANOVA test using the SPSS software.

**Disclosure statement**

No potential conflict of interest was reported by author(s).

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