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Article

Transgenic Phenotypic Differences of Grapevine Circular RNA between *Arabidopsis* and Grapevine Callus Imply the Species-dependent Functions of *Vv-circPTCD1*

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Abstract: Circular RNAs (circRNAs) served as covalently closed single-stranded RNAs have been proposed to influence plant development and stress resistance. Grapevine is the most economically valuable fruit crops cultivated worldwide and threaten by various abiotic stresses. Herein, we reported that a circRNA (*Vv-circPTCD1*) processed from the second exon of a pentatricopeptide repeat family gene *PTCD1* was preferentially expressed in leaves and responded to salt and drought but not heat stress in grapevine. Additionally, the second exon sequence of *PTCD1* was highly conserved but the biogenesis of *Vv-circPTCD1* is species-dependent in plants. It was further found that the overexpressed *Vv-circPTCD1* can slightly decreased abundance of the cognate host gene and the neighboring genes were barely affected in grapevine callus. Furthermore, we also successfully overexpressed the *Vv-circPTCD1*, and found that the *Vv-circPTCD1* deteriorated the growth during heat, salt, and drought stresses in *Arabidopsis*. However, the biological effects on grapevine callus were not always consistent with that of *Arabidopsis*. Interestingly, we found that the transgenic plants of linear counterpart sequence also conferred the same phenotypes as that of circRNA during the three stress conditions, no matter what species. Those results imply that although the sequences are conserved, the biogenesis and functions of *Vv-circPTCD1* are species-dependent. Our results indicate that the plant circRNA function investigation should be conducted in homologous species, which support valuable reference for further plant circRNA studies.

Keywords: grapevine; abiotic stress; non coding RNA; CircRNA; pentatricopeptide repeat proteins; back-splicing

1. Introduction

Noncoding RNAs have increasingly crucial functions for growth and development and stress response in plants [1, 2]. Circular RNAs (CircRNAs), which has not been found the coding ability in plants yet, are single strand non-coding RNA that are processed by backing-splicing, in which a downstream 5' splice site is linked by 3',5'- phosphodiester bond to an upstream 3' splice site in a reverse order crossing one exon or exons [3]. Owing to the development of high-throughput sequencing technology and associated analytical tools, thousands of circRNAs have been identified in various plants and were deposited in associated database [4, 5]. At present, it has been reported that a total of 171,118 circRNAs from 21 plant species have been collected in PlantcircBase [6]. The biological functions of circRNA have been well investigated in mammals. Animal circRNAs have generally been suggested to function as miRNA sponges for decaying endogenous miRNA [7], as templates for translation [8, 9], as mediator for regulating assembly and activation of AMPK complex

under stress [10], as enhancer for *cis*-regulation of their host genes [11]. In plants, previous studies have focused on identification and annotation of putative circRNAs as well as function investigation. Until now, many studies have provided direct evidences that circRNAs shown important functions in plants by overexpression and gene editing technologies. Overexpression of circRNA derived from *PSY1* (*Phytoene Synthase 1*) and *PDS* (*Phytoene Desaturase*) in tomato, respectively, causes color variation [12]. In *Arabidopsis*, *circSEP3* from exon 6 of *SEPALLATA3* can regulates alternative splicing of its host genes through R-loop formation with homological genomic region. Overexpression of this circRNA results in floral organ abnormality [13]. CRISPR-Cas9 strategy has been applied to remove *Os06circ02797* processing by editing the flanking sequence in rice, resulted in a rapid growth phenotype and higher chlorophyll A/B content [14]. In *Populus tomentosa*, overexpression of *Circ_0003418* derived from a RING-type E3 ligase gene *XBAT32* deteriorates the callus thermotolerance [15]. Although some studies have been performed, functional investigation on circRNAs in plants is still very limited. Therefore, additional researches are further conducted.

Grapevine (*Vitis spp.*) is the most economically important fruit crops cultivated worldwide, for the purpose of producing table grape, dried fruits, and as the raw materials for juice and wine making [16]. The growth, development and flavor of berries are strongly influenced by various abiotic stresses, such as high temperature, water deficit, and salt during viticulture [17-19]. Previous findings suggested that high temperature influences grapevine photosynthesis, florescence and veraison [20-22]. In grapevine, the serine/arginine-rich splicing factors (SR) are generally phosphorylated on the condition of high temperature, which results in the disturbance of alternative splicing of pre-mRNA [23, 24]. The mechanisms involved in grapevine tolerance to high temperature are poorly understood, although the heat shock factors (HSFs)-mediated regulatory networks have been well deciphered in other plants [25-27]. Drought is another important environment factor limiting grapevine growth and productivity, and studies have shown that the photosynthesis of vegetative organs, berries size and yield, and accumulation of secondary metabolites are greatly affected by the water deficit [28]. The abscisic acid (ABA) is induced and plays a key role for response to water deficit by regulating stomatal movements, as well as the signaling pathway has been well studied in plants [29]. Furthermore, the long non coding RNAs (LncRNA) and microRNAs (miRNA) are also serves as novel regulators of the plant response to drought stress in *Arabidopsis* [30], *Brassica napus* L [31], and grapevine [32]. Salt stress is another factor which is mainly due to the hyperosmotic conditions of soil solution with increase of Na⁺ and Cl⁻, causing the deficiency of the absorption of water and nutrients in plants [33]. In grapevine, previous studies found that many stress-induced genes, such as *VvWRKY2*, *VvNAC17*, and *VvASMT1*, perform functions for enhancing salt and osmotic stresses tolerance in ectopic overexpression [19, 34, 35]. Although the above findings greatly uncover the molecular mechanisms responding to abiotic stress in grapevine, the function of circRNAs also need to be further explored.

In grapevine, according to our previous study, a total of 8,354 circRNAs have been identified by the whole transcriptome analysis, and a *Vv-circATS1* spliced from *glycerol-3-P acyltransferase* was proved to improve cold tolerance in *Arabidopsis* [36]. In present study, a circRNA derived from the second exon of the *pentatricopeptide repeat domain-containing protein 1* (*PTCD1*, VIT_211s0016g03830) was verified based on the grapevine circRNA dataset, named *Vv-circPTCD1*. Pentatricopeptide repeat (PPR) proteins is a large gene family in plants, which are characterized by 2 to 15 tandem repeats of 30-40 amino acid length motif. Some PPR proteins have been supposed to play roles in organellar RNA metabolism, organ development as well as stress response [37]. Overexpression of *Vv-circPTCD1* deteriorated the tolerance to heat, salt, and PEG-mediated osmotic stresses in *Arabidopsis*. While, similarly, the linear fragment identical with the circRNA sequence also did. However, the *Vv-circPTCD1* overexpressed callus mass of 'Thompson Seedless' were failure to display the phenotypes consistent with *Arabidopsis*. Those results implied that the functions of *Vv-circPTCD1* potentially were sequence-dependent and not conserved among plants.

2. Results

2.1. Identification and verification of *Vv-circPTCD1*

Based on the previous published circRNA dataset of ‘Muscat Hamburg’ [36], a circRNA_4363, back-spliced from the second exon of a pentatricopeptide repeat (PPR) proteins family gene *PTCD1*, was identified and renamed *Vv-circPTCD1* in this research. The *PTCD1* encodes a protein with 636 amino acid residues length and contains the 9 tandem PPR domain (Figure 1A). In *Arabidopsis* genome, meanwhile, two orthologs (AT5G21222 and AT5G25630) were characterized and phylogenetic analysis showed that AT5G21222 and *PTCD1* (VIT_211s0016g038030) were clustered into one clade with 96% confidence (1000 replicates) (Figure 1B). The *Vv-circPTCD1* were further verified by cloning and sequencing as well as the back-splicing site was AG/GA (Figure 1C). Divergent and convergent primers were designed to amplify the target fragment form DNA and leaf cDNA, result showed that the fragment with the back-splicing site was only detected in cDNA templates (Figure 1D).

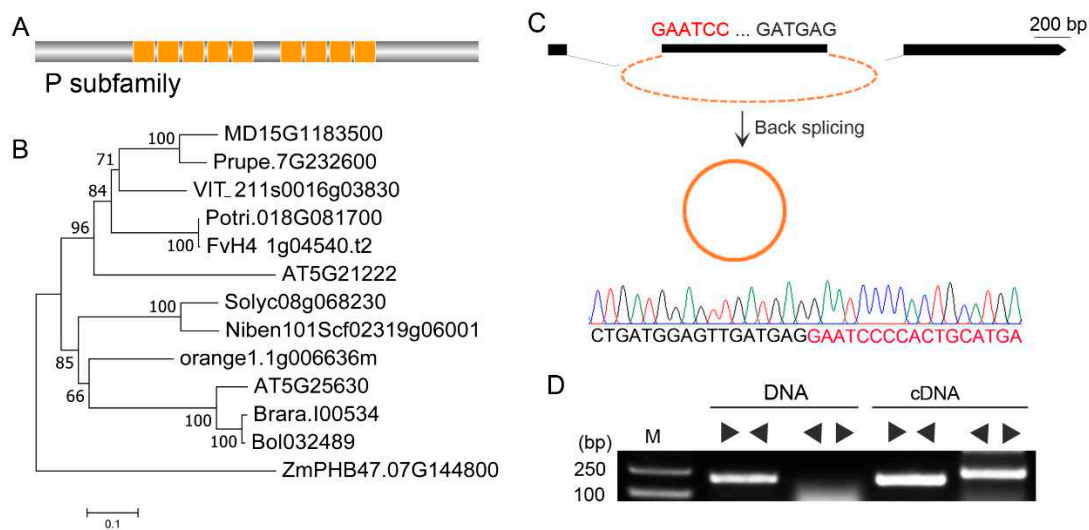


Figure 1. Identification of *Vv-circPTCD1* in grapevine. (A) The graphical representations of *PTCD1* protein; (B) The phylogenetic analysis of *PTCD1* (VIT_211s0016g038030) with orthologs in other species. (C) Verification of *Vv-circPTCD1* processed in the second exon. (D) PCR amplification of divergent and convergent primers based on DNA and cDNA in grapevine. “▶◀” indicated the convergent primer pairs, “◀▶” indicated the divergent primer pairs.

2.2. Nonconservative back-splicing of *Vv-circPTCD1* in plants

To investigate whether the *Vv-circPTCD1* was conserved in plants, we analyzed the physical gene structure of *PTCD1* in plants, including monocots and dicots. The result showed that similar physical gene structure of *PTCD1* orthologs was observed (Figure S1), as well as a CDS (coding sequence) back-spliced into *Vv-circPTCD1* in grapevine was found and it was highly conserved by multiple sequence alignment among plants (Figure S2). It was reasonably hypothesized that this circRNA orthologous with *Vv-circPTCD1* maybe also conserved in plants. Therefore, the divergent primer pair was tentatively designed to clone the orthologs in *Arabidopsis* and *N. benthamiana*, although the non-specific amplification was found in *N. benthamiana*, while the *Vv-circPTCD1* orthologs were failure to cloned (Figure S3). Those results suggested that the back-splicing of *Vv-circPTCD1* potentially was species-dependent.

2.3. The expression analyses of *PTCD1* and *Vv-circPTCD1* under abiotic stress

To investigate the potential functions in grapevine, we analyzed the tissue specificity and the expression patterns of *Vv-CircPTCD1* and its corresponding host gene under salt, heat, and drought stress conditions, respectively. The *Vv-CircPTCD1* was mainly expressed in young and mature leaves while scarcely expressed in roots, consistent with that of host gene (Figure 2A). However, the accumulation level of host gene was excessively higher than that of *Vv-CircPTCD1* (more than sixty times) (Figure 2A). During salt stress, in addition to the host gene was mildly down-regulated during initial treatment, the *Vv-CircPTCD1* was significantly down-regulated on the fifth day after treatment (Figure 2B, C). During drought treatment, the *VvSnRK2I* (VIT_207s0197g00080), a marker gene homologous with *AtSRK2I/AtSnRK2.3* induced by ABA in *Arabidopsis* [42], was significantly induced when the soil water potential reduces to -0.8 and -1.0 MPa (Figure 2D). The expression level of *PTCD1* was mildly up-regulation when the water potential was -0.4 MPa, while the expression level of *PTCD1* was significantly reduced when the water potential was reduced to -0.8 and -1.0 MPa (Figure 2E). However, the expression pattern of *Vv-CircPTCD1* was barely changed during drought treatment (Figure 2F). We further investigated the expression pattern during heat stress, the results showed that the heat shock factor genes *VvHsfA7*, as a marker of heat stress [20], were remarkable up-regulation (Figure 2G) and the expression level of *PTCD1* was down-regulation with the prolongation of treatment (Figure 2H). Although the expression level of *Vv-CircPTCD1* was down-regulation after 4 h treatment, however, the expression level was also slightly up-regulation after 2 h treatment (Figure 2I). Those results indicated that the *Vv-CircPTCD1* expression was always fell behind that of *PTCD1* and potentially play a role during abiotic stresses in grapevine.

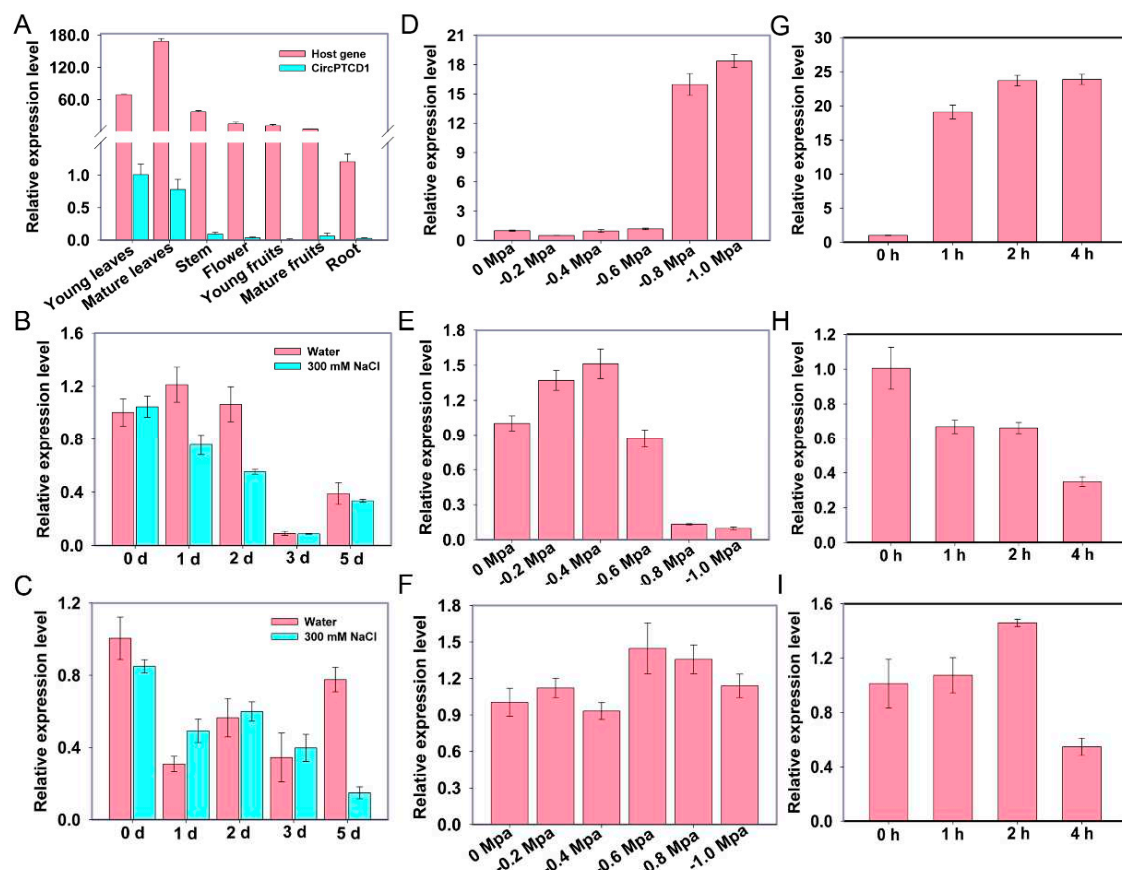


Figure 2. The expression patterns of *PTCD1* and *Vv-circPTCD1* under stress. (A) The tissue-specificity analyses of *PTCD1* and *Vv-CircPTCD1*; (D, G) The expression pattern of marker genes *VvSnRK2I* and *VvHsfA7* under drought and heat treatment; (B, E, H) The expression pattern of *PTCD1* during salt, drought, and heat stress; (C, F, I) The expression pattern of *Vv-CircPTCD1* during salt, drought, and heat stress.

2.4. Overexpression of *Vv-CircPTCD1* in grapevine callus and *Arabidopsis*

To decipher the functions of *Vv-CircPTCD1* in plants, the vector for *Vv-CircPTCD1* overexpression (OE) was generated, meanwhile, corresponding linear fragment (Linear_PTCD1) identical with *Vv-CircPTCD1* was also constructed as a control (Figure 3A). The overexpressed callus mass was verified by RT-PCR and RT-qPCR, respectively (Figure 3B, C, D, E). The expression level of host gene and flanking genes were further investigated in all OE callus mass. Among *Vv-CircPTCD1* lines, the expression level of host gene was slightly down-regulated (Figure 3F), while the up-stream gene (VIT_211s0016g03850) and down-stream gene (VIT_211s0016g03820) were not significantly affected (Figure 3G, H). However, among Linear_PTCD1 OE lines, the expression level of host gene was discrepant among different lines, which is down-regulated in line 2 (Figure 3I). As well as the up-stream gene and down-stream gene were also slightly down-regulated (Figure 3J, K). In order to uncover whether the function of *Vv-CircPTCD1* is conserved among plants, simultaneously, ectopic transformation was also performed in *Arabidopsis* (Figure S4).

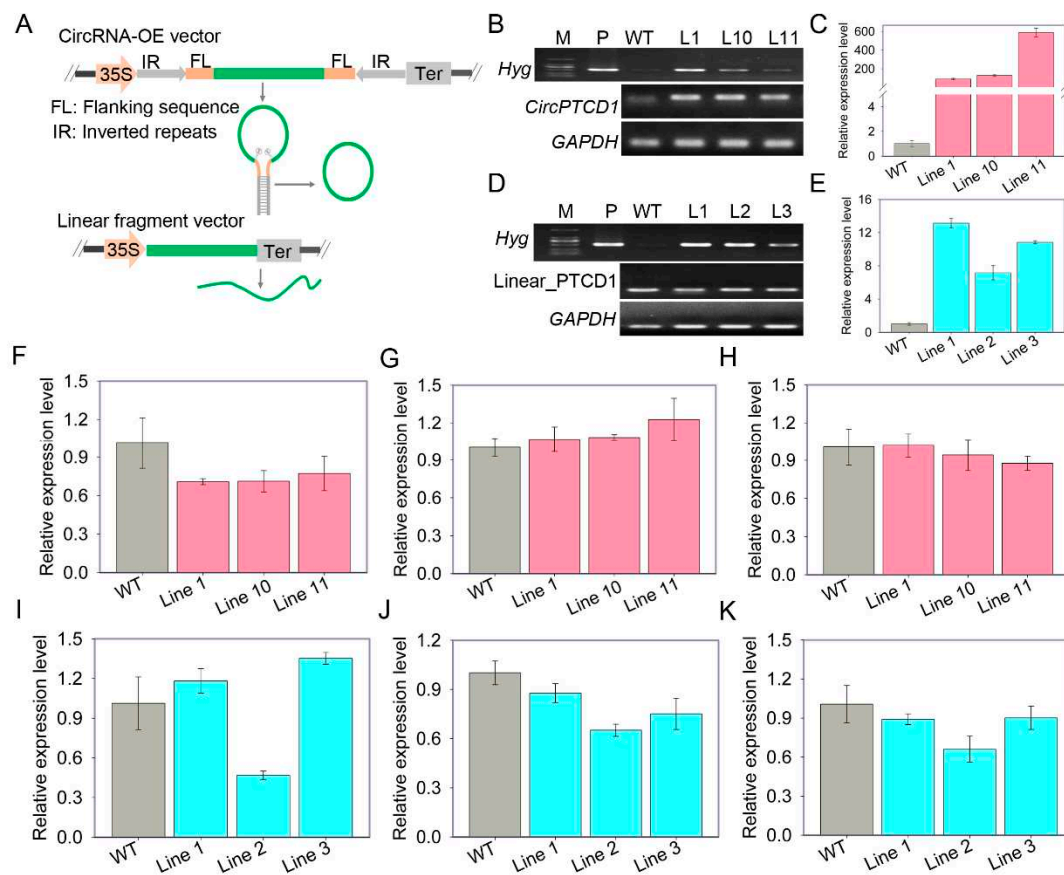


Figure 3. The OE of *Vv-CircPTCD1* and corresponding linear fragment (Linear_PTCD1) in callus of ‘Thompson Seedless’ (A) the schematic of vector construction for circRNA and linear fragment OE, the green line represents the sequence back-spliced into circRNA and the CaMV35S (35S) promoter was used to initiate transcription, Ter refers to transcription termination; (B, C) the PCR and RT-qPCR confirmation of *Vv-CircPTCD1* OE in grapevine callus, three positive callus mass were identified, *Hyg* encode the hygromycin phosphotransferase responsible for resistance selection; (D, E) the PCR and RT-qPCR confirmation of Linear_PTCD1 OE in grapevine callus; (F, G, H) the expression level of host gene, upstream gene and downstream gene in *Vv-CircPTCD1* OE lines; (I, J, K) the expression level of host gene, upstream gene and downstream gene in Linear_PTCD1 OE callus mass.

2.5. Phenotype of OE lines under heat stress

To investigate whether *Vv-CircPTCD1* plays a role in *Arabidopsis*, the four-weeks old seedlings were treated 12 h at 45°C followed by exposure to 22 °C for 7 days to recovery. Results showed that, comparing with WT, the rosette leaves were significantly deteriorated in *Vv-CircPTCD1* OE lines (Figure 4A,B). It was a wonder that the rosette leaves were also remarkably damaged in linear fragment OE lines, which keep an identical sequence with *Vv-CircPTCD1* (Figure 4A, B). We also observed the phenotype of transgenic callus responding to heat stress, in which the callus was continuously incubated 15 d at 40°C. However, phenotypic differences were nonevent, no matter which OE callus mass (Figure 4C and Figure S5).

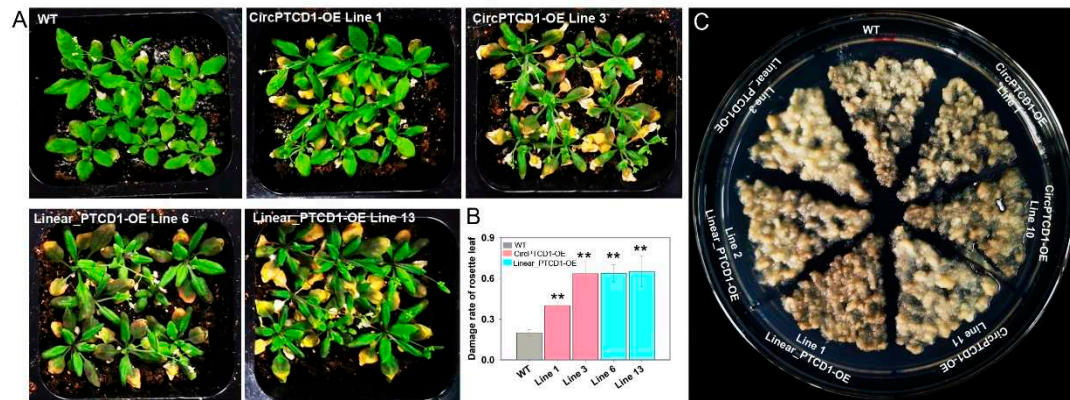


Figure 4. The phenotype of OE lines in *Arabidopsis* and callus of grapevine under heat stress (A) Deteriorated phenotype of transgenic *Arabidopsis*; (B) damage rate of rosette leaves in overexpressed *Arabidopsis*; (C) the phenotype of overexpressed callus under heat stress. Differences between mean values of groups were compared using *t*-test (***: $p < 0.01$).

2.6. Phenotype of OE lines under salt stress

To further survey the function of *Vv-CircPTCD1* in plants, the four days old seedlings of *Arabidopsis* were transplanted into normal and NaCl-containing medium to observe the variation. Results suggested that different OE lines and WT seedlings were normally and identically grow in NaCl-free medium (Figure 5A), while the survival rate of transgenic lines were lower on the NaCl-containing medium after five days compared to WT (Figure 5B, C). In grapevine callus, the OE callus mass transformed linear fragment adversely displayed albino phenotype, while the OE callus mass transformed *Vv-CircPTCD1* was also did but much slighter compared to WT callus mass (Figure 5D). Those results also showed the *Vv-CircPTCD1* potentially served as negative factor for salt stress and the linear fragment identical with the *Vv-CircPTCD1* also plays the same roles in *Arabidopsis* and grapevine callus.

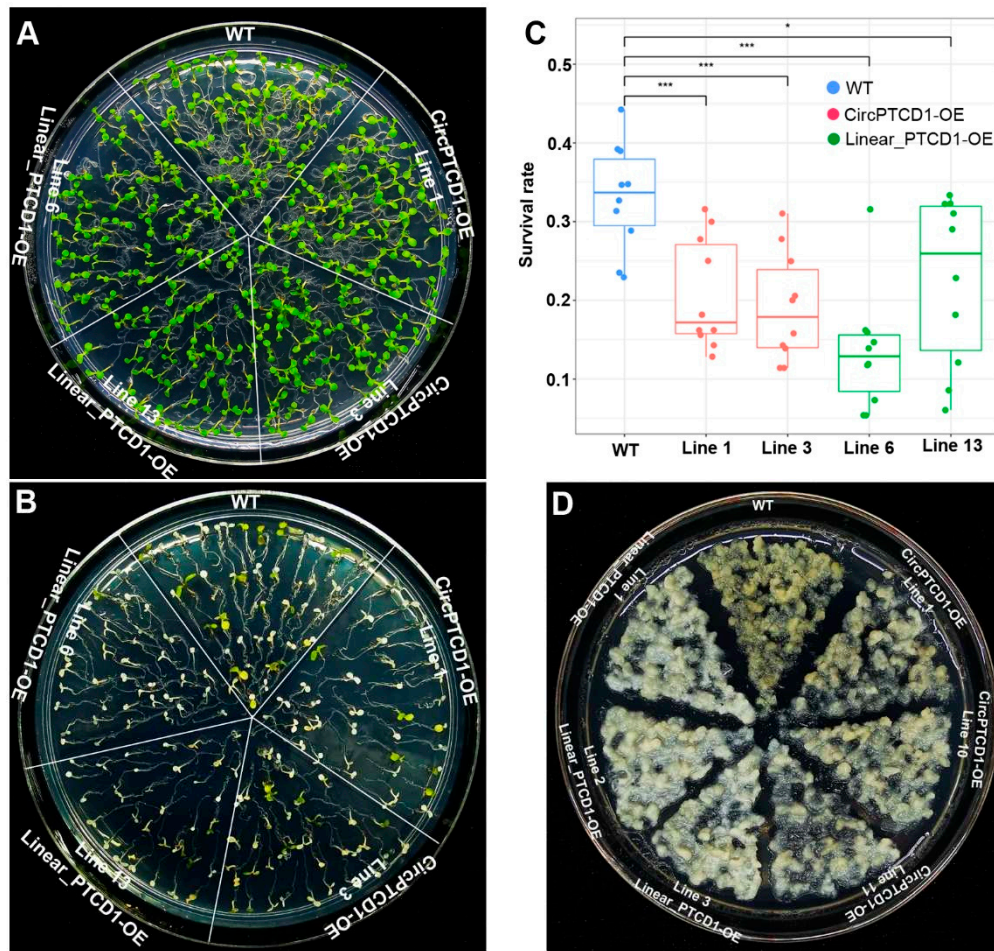


Figure 5. The phenotype of OE lines in *Arabidopsis* and callus of grapevine under salt stress (A) The phenotype of OE seedlings and WT on 1/2 MS medium; (B, C) The phenotype and survival rate of OE seedlings and WT on 1/2 MS medium containing 300 mM NaCl; (D) The phenotype of OE and WT callus mass incubated on medium containing 300 mM NaCl. Differences between mean values of groups were compared using *t*-test ("": $p < 0.05$; "": $p < 0.001$).

2.7. Phenotype of OE lines under drought stress

To comprehensively explore the function of *Vv-CircPTCD1* in *Arabidopsis*, the four weeks old seedlings of *Arabidopsis* were stopped watering. Results showed that the transgenic lines were much more susceptible and withering (Figure 6A). The four days old seedlings were further transplanted into normal and PEG-containing medium, and found that different transgenic lines and WT seedlings were normally and identically grow in normal medium (Figure 6B), while the growth of rosette leaves were obviously inhibited in OE lines but the growth of root barely did (Figure 6C, D, E). Those results suggested that the *Vv-CircPTCD1* and corresponding linear fragment also play negative roles during drought stress in *Arabidopsis*. In grapevine callus, oppositely, the OE callus mass of transgenic callus mass significantly displayed resistance to osmotic stress mediated by PEG-8000, while the WT has become brawn after 30 days incubation (Figure 6F). We further counted the growth index of callus mass, and found that the growth rate of transgenic callus was significantly higher than that of WT (Figure 6G and Figure S5). Those results implied that the function of *Vv-CircPTCD1* potentially is species-dependent.

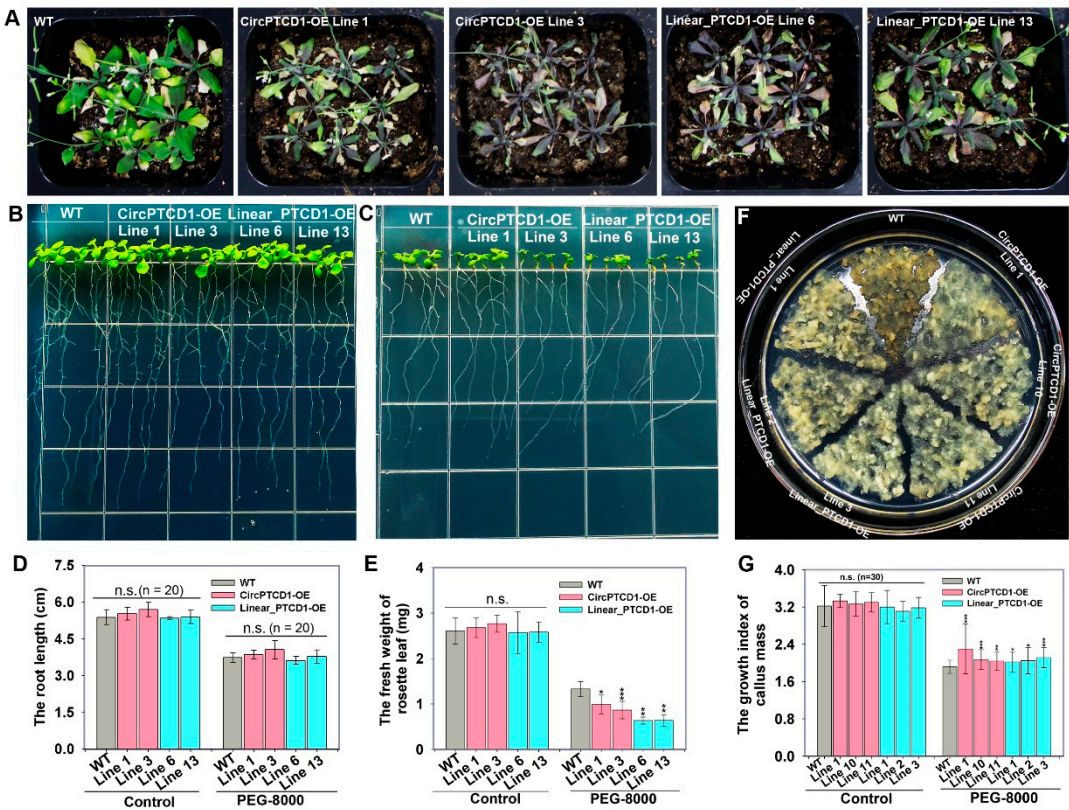


Figure 6. The phenotype of OE lines in Arabidopsis and callus of grapevine under drought stress (A) The phenotype of a mouth old Arabidopsis during drought stress; (B) the growth of seedlings incubated on PEG-free medium; (C) the growth of seedlings incubated on medium containing PEG-8000; (D, E) the root length and fresh weight of rosette leaves of seedlings during PEG-8000 treatment; (F) the phenotype of grapevine callus mass incubated on medium containing PEG-8000; (G) the growth index of callus mass incubated on medium containing PEG-8000. Differences between mean values of groups were compared using *t*-test ("*": $p < 0.05$; "***": $p < 0.01$; "****": $p < 0.001$; n.s.: $p > 0.05$).

3. Discussion

CircRNAs are widespread in plants. Most circRNAs are generated by backing-splicing from the exon or exons of genic primary mRNA, for example 94.5% are derived from the coding genes in rice and the remaining were intergenic circRNAs [43]. In addition, the mitochondrion genome is also proved to generate circRNAs, called mitochondrion-encoded circular RNAs (mcircRNAs) [44]. Here, we identified a circRNA spliced from the second exon of *PTCD1*, a PPR family genes, in which it was spliced based on canonical GU/AG splicing signal [36] (Figure 1C). In general, hundreds of this superfamily proteins encoded by the PPR family genes located in the nuclear and organelle genome are found in plants and characterized by multiple tandem arrays of 35 residues PPR domain [45]. Previous finding proved that the PPR proteins, served as RNA binding protein, affect cytoplasmic male sterility, embryogenesis, seed development, and stress [46-49]. Interestingly, the physical gene structure of *PTCD1* orthologs are similar, as well as the sequence of the CDS back-spliced into *Vv-circPTCD1* are highly conserved among plants (Figure S1 and S2). However, the verification failure of corresponding circRNA in *Arabidopsis* and *N. benthamiana* imply that the *circPTCD1* is not naturally conserved among plants. No matter what mammals and plants, a factually small proportion of circRNAs are supposed to be conserved, such as the overlap of 26% circRNAs in both human and mouse based on the syntenic region of genome [8]. In plants, only 8.7% circRNAs are genomic conservation in dicotyledonous plants, even that are less than half in *Oryza* genus [43]. Taken together, the *Vv-circPTCD1* is not evolutionarily conserved, although the host genes are conserved. And this result partially supports the idea that circRNAs result largely from splicing errors [50, 51].

We also successfully constructed the artificial vector containing a reverse complementary sequence pairs to generate the circRNA in *Arabidopsis* and grapevine callus according to the previous strategy, although the natural back-splicing of *Vv-circPTCD1* is not conserved (Figure 3A) [36]. The intronic complementary sequences have been found in the flanking sequence of highly expressed circRNA in eukaryotes. In mammals, the exons flanked by the orientation-opposite *Alu* elements, a shorted interspersed elements in primates, are preferentially circularized [52]. In *Populus tomentosa*, the miniature inverted repeat transposable elements (MITEs) are enriched in circRNA flanking regions and served as *cis*-regulatory factor to significantly regulate the exon circularization [15]. Furthermore, previous finding supposed that the back-splicing of circRNA is also executed by the canonical spliceosome, a highly conserved precursor mRNA splicing mechanism in eukaryotes [53]. Additionally, the flanking intron sequence is crucial adjective for precise splicing and circularization of one exon and exons, which has been proved by the heterologous investigations [36, 54]. Therefore, the inverted repeats and flanking sequence are commonly contributed to the fidelity and expression level of *Vv-CircPTCD1* in *Arabidopsis* and grapevine callus (Figure 3B, C and Figure S4). Therefore, the artificial “stem-loop” structure mediated by the reverse complementary sequence greatly contributes to the back-splicing of *Vv-CircPTCD1* in heterogeneous expression, in which the “stem-loop” brings the splice sites into close proximity to each other [55].

Additionally, previous investigations uncovered that circRNAs are incorporated into chromatin by R-loop structure interacting with the nuclear DNA to regulate the chromatin stabilization or alternative splicing [13, 56]. The host gene and neighboring genes are potentially influenced thorough overexpressed or deleted circRNA. In rice, a neighboring gene is significantly up-regulated at circRNA mutant lines [14]. In this study, the *Vv-CircPTCD1* has slightly negative effect on the host gene, and barely do on the neighboring genes after overexpression of *Vv-CircPTCD1* (Figure 3F, G, H). In *P. tomentosa*, overexpression of *Circ_0003418* significantly increased the expression abundance of its parental gene [15]. Therefore, the circRNA potentially have the ability to function through disturbing the expression of host or neighboring genes in plants.

The expression pattern of circRNAs is also determined by tissue and environment factors, and even genotype [15, 57]. We found that the *Vv-CircPTCD1* focused in this research were preferentially expressed in young and mature leaves, which is consistent with the expression pattern of corresponding host gene, while the expression abundance of *Vv-CircPTCD1* greatly less than 2% that of host gene (Figure 2A). In addition to some circRNAs in animals (almost 10%) [8], the circRNAs barely hold a higher expression level with respect to the linear counterpart and are largely obeyed the expression pattern of host gene in plants, such as grapevine [36]. In this study, the expression level of *PTCD1* is significantly inhibited during drought stress, while the expression of *Vv-CircPTCD1* is slightly decreased (Figure 2D, E, F), indicating it plays a role in grapevine responding to drought stress. In addition, the expression level of *Vv-CircPTCD1* is remarkably decreased during salt stress after 5 days, while the host gene never occurred (Figure 2B, C). Those results imply that the *Vv-CircPTCD1* plays a role during abiotic stress, in which the expression of circRNA potentially is controlled on the condition of specific abiotic stress, at least during the processing or degradation pathway [58].

The hypothesis of “splicing error” is indeed uncontradictory with the biochemical activities and biological functions of circRNAs. According the previous idea, the biogenesis of circRNAs and the regulation of their abundance are managed by various mechanisms, such as *cis*- or *trans*-elements, export and turnover of circRNAs [3]. In this study, although the expression of *Vv-CircPTCD1* is related to the salt and drought stress, the mechanism is also unclear. For investigation of the biological function, a problem with their circular conformation and sequence overlap with linear cognate mRNAs and incomplete cyclization is difficult to overcome [53]. The strategy to evade this problem is to transform the linear fragment identical with circRNA as a control. Generally, the linear fragment scarcely functions in plants [13, 15, 36]. In present research, the linear counterpart is also transformed as a control (Figure 3D, E and Figure S4). Interestingly, identical stress-induced phenotype is observed between *Vv-CircPTCD1* and its linear counterpart OE lines (Figure 4, 5, 6). It is putative that (1) the incompletely cyclized transcript plays roles identical with linear counterpart;

(2) the *Vv-CircPTCD1* functions through sequence-dependent style. We observed that only the phenotype induced by salt stress is conserved between *Arabidopsis* and grapevine callus (Figure 5), and that is distinctly inconsistent during the treatment of heat and PEG (Figure 3 and Figure 6). Coincidentally, although the cognate sequence generated *Vv-CircPTCD1* are greatly conserved among multiple species (Figure S2), while the biogenesis is not conserved (Figure S3). Those results imply that the biological functions of circRNA are potentially species-dependent in plants.

4. Materials and methods

4.1. Plant materials and treatments

For drought treatment of grapevine, 1-year-old 'Muscat Hamburg' cuttings were planted in a mixture of loam and sand (1:1, v/v) in flowerpot in a greenhouse. Cuttings with four well-developed leaves were drought stress treated. The well-developed leaves were collected when the soil water potential was 0 MPa, -0.2 MPa, -0.4 MPa, -0.6 MPa, -0.8 MPa, and -1.0 MPa, respectively. For heat stress treatment of grapevine, the cuttings were treated at 45 °C, and the leaves were collected at 0 h, 1 h, 2 h, and 4 h, respectively. For salt stress, the cuttings were planted in a chamber (25 °C) and watered the 300 mM NaCl solution, and the mature leaves were sampled at 0 d, 1 d, 2 d, 3 d, and 5 d. All samples were quickly frozen in liquid nitrogen and then stored at -80 °C. All treatments were carried out in three biological replicates.

Arabidopsis ecotype Col-0 was cultured in chamber under 16 h light/8 h dark conditions with a light intensity of 600 mmol m⁻² s⁻¹ at a temperature of 22°C. For heat stress treatment of *Arabidopsis*, 6 weeks-old WT and transgenic plants were exposed to heat stress at 45°C for 12 h followed by exposure to 22°C for 7 days to recovery. For drought stress treatment, 6-weeks-old WT and transgenic plants were stopped watering until the substrate is completely dry, and observed the phenotype. The PEG-8000 treatment (the water potential was -0.7 MPa) was performed according to the Verslues's descriptions [38]. And the 4 days old seedlings were transferred into the plate containing PEG-8000 and cultured in chamber for another 5 days. For salt treatment, the plates containing 1/2 MS salts supplemented with 300 mM NaCl were prepared and 4 days old seedlings were incubated. The survival rates were counted after 5 days.

4.2. Validation of circRNA and RT-qPCR

The total RNA of *Arabidopsis* and grapevine callus was extracted by TRIzol reagent (Sangon Biotech, China). And the total RNA of grapevine leaves was extracted by CTAB method. In brief, 200 mg sample are powered and transferred into RNase-free 2 mL tube. Added quickly 900 µL pre-heated (65 °C) CTAB buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 2 M NaCl, 2% PVP-40, pH8.0) and 20 µL β-mercaptoethanol. Mixed the mixture robustly and then bathed at 65°C for 15 min. Extracted lysate with 900 µL of chloroform: isoamylol (24:1, v/v) by vortexing for 30 s and centrifuged at 4 °C for 10 min at 12,000 rpm. Repeated two times with chloroform: isoamylol extraction. Added 20 µL NaAC (pH5.2) and 60 µL pre-cooled (-20°C) absolute alcohol, and bathed 10 min in ice. And added volume chloroform: isoamylol for vortexing. After centrifuged at 4°C for 10 min at 12,000 rpm, added 150 µL 10 M LiCl to 450 µL supernatant and mixed. The RNA was precipitated at 4°C for 6 to 8 h, and harvested by centrifugation at 4°C for 10 min at 12,000 rpm. Carefully removed the supernatant, and washed two times by 70 % pre-cooled (-20°C) alcohol. The pellet was dried and resuspended by 30 µL DEPC-treated ddH₂O.

The total RNA was transcribed to cDNA using random primers by a FastKing RT Kit (TIANGEN, China) in accordance with the manufacturer's instructions. To confirm the grapevine circRNAs predicted, divergent primer was designed by Primer 5 (Table S1). PCR was as follows: 94°C for 3 min; 40 cycles at 94°C for 30 s, 56°C for 15 s, and 72°C for 20 s; and then 1 cycle at 72°C for 5 min. For PCR, 2×Taq master mix (Vazyme, China) was used. The PCR products were separated by agarose gel electrophoresis and then purified. Sanger sequencing was further performed to verify the back-spliced junction sites. RT-qPCR (real-time quantitative PCR) analysis was conducted to evaluate the expression levels of circRNAs and linear counterparts using Talent qPCR PreMix (TIANGEN,

China) with a qTOWER³ Real-Time PCR Detection System (Analytikjena, Germany). Gene expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method [39].

4.3. Vector construction

All expression vectors were constructed based on the pHB binary plasmids. The construction of circRNA expression vector was conducted based on previous published strategy [36]. For linear RNA expression vector, the linear fragment derived from the same sequence with circRNA was cloned into pHB vector which was digested by *Bam*H I and *Xba* I. The constructed plasmids were individually transformed into *Agrobacterium tumefaciens* GV3101 strains and incubated at 28°C for 2-3 d on the LB medium supplemented with 25 mg·L⁻¹ rifampicin (Rif) and 50 mg·L⁻¹ kanamycin (Kan). A single clone was incubated at 200 rpm and 28°C for 8-10 h in LB liquid medium containing 25 mg·L⁻¹ Rif and 50 mg·L⁻¹ Kan. The presence of the corresponding plasmid was verified by PCR. The positive clones were added glycerol and stored at -80°C.

4.4. Arabidopsis transformation and verification

Arabidopsis transformation was performed according to floral dip method [40]. T1 seedling were selected by spraying of Glufosinate ammonium 10% solution (dilute 1000×) (Sangon Biotech, China). The positive transgenic ones were transferred into pots and confirmed by PCR detection using the gene primers. For circRNAs confirmation, the cDNA was used as templates for PCR and RT-qPCR reaction and the splicing site was verified by further sequenced.

4.5. Callus transformation of 'Thompson Seedless' and treatment

The embryogenic callus was induced from floral explants of 'Thompson Seedless' according to a previously published protocol [41]. The callus mass was monthly sub-cultured in MSTP medium (MS base salts, 20 g·L⁻¹ sucrose, 1 mg·L⁻¹ TDZ, 2.2 mg·L⁻¹ picloram, pH5.8) in dark condition at 26°C. For callus transformation, the *A. tumefaciens* containing vector was inoculated in 20 mL LB liquid medium and incubated until OD₆₀₀ reached 1.0. After centrifugation for 5 min at 6000 rpm and 25°C, the medium was discarded. The pellet was resuspended and the OD₆₀₀ was adjusted to 0.4 with MS medium (0.1 mM acetosyringone, pH5.8). The bacterial mixture was incubated with the embryogenic callus for 30 min at 40 rpm in 50 mL conical flask, and was then transferred onto filter paper to fully remove and evaporate excess liquid. Then, the callus was collected and transferred to solid MS medium (0.1 mM acetosyringone, 8g·L⁻¹ agar, pH5.8) and co-cultivated in dark condition at 26 °C for 3 days. After 3 days, the callus was transferred to solid MSTP medium containing 50 mg·L⁻¹ hygromycin, 200 mg·L⁻¹ Timentin. Petri dishes were monthly sub-cultured in the dark for the induction of transgenic callus. The expression of circRNA in transgenic callus was confirmed by PCR and RT-qPCR using divergent primer pairs.

For heat stress treatment, the callus mass was sub-cultured into MSTP medium for one week at 26 °C and then further cultured at 40 °C for 15 days. The callus mass was sub-cultured into MSTP medium supplemented with 300 mM NaCl for salt stress at 26 °C. For PEG-8000 treatment, the plates were prepared according to above description, of which the base salt was replaced by MSTP medium supplemented with 15 g·L⁻¹ agar on solid layer but the liquid layer was the MSTP medium without sucrose and agar. Then the callus mass was sub-cultured into the PEG-containing plates and cultured at 26 °C for one month.

5. Conclusions

According to this result, Although the cognate counterpart mRNA is greatly conserved among plants, the biogenesis of circRNA generated from them potentially is not. Furthermore, the biological effects of circRNA are largely species-dependent.

Author Contributions: CM designed the experiments and revised the manuscript; YR performed the callus transformation, phenotype collection, and drafted the manuscript; JL and ZZ conducted the callus induction and

sub-culture; YS, DF, ML, LZ, YX, and DG performed sample treatment, Arabidopsis transformation, and vector construction; JH, SS, and ZG contributed to the data collection and analyses.

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