Cold Resistance in the Callus of *Vitis Vinifera* 'Zaoxia' Was Enhanced by Overexpressing the *VyDHN1* Gene

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Abstract. The cold resistance of grapevine (*vitis vinifera* L.) which is one of the most important economic fruit trees in the world is low. The grown distribution of grapevine was restricted by climate and other factors. Dehydrins (DHN) are a class of proteins that play an important role in plants' defense against stress. The VyDHN1 was cloned from *V. yeshanensis* which is highly resistant to cold stress, but the function and related mechanism of this gene in response to cold stress in grapevine has not been elucidated yet. In this study, the genetic transformation system was successfully established using callus induced by exocarp tissue of table grape varieties (*V. vinifera* 'Zaoxia' and '87-1') by agrobacterium-mediated method. The *VyDHN1* gene was stably overexpressed into the callus and the physiological indicators of the transgenic callus were detected under cold stress. The study revealed that *VyDHN1* overexpression can maintain the cell membrane stability of the transgenic callus of Zaoxia during cold stress which could improve the cold resistance of grapevine.

Keywords: *VyDHN1*, callus, genetic transformation, cold resistance.

1. Introduction

Low temperature is a prominent abiotic stress that imposes limitations on plant growth, development, and geographical distribution[1]. This stress induces rigidity in the cell membrane, disrupts protein structure and function, and hampers various physiological functions such as antioxidant activity and photosynthesis[2, 3]. Nonetheless, cold damage might cause plants in temperate regions to develop low-temperature acclimation mechanisms, increasing the plants' resilience to frost[4]. According to research, plants often create and store a type of protein called dehydrin (DHN), which is prevalent in late embryonic development and is a member of the LEAII family, in response to stress in order to increase plant tolerance to stress[5]. It is crucial to the process of the capacity of a plant to withstand stress.

Grapevine has a limited response to stress from low temperatures, and its genetic background is somewhat complicated. Traditional breeding is taken a long time since environmental factors like season and geography easily hinder the improvement of varieties. As grapevine tissue culture technology advances, it becomes increasingly important to establish an effective regeneration system, carry out genetic transformation, and use this method for genetic breeding[6].

There are two categories for the grapevine regeneration system: the embryogenesis pathway and the organogenesis pathway[7]. Somatic embryos have a laborious and lengthy induction process. However, in this regeneration process, the low germination rate of somatic embryos is an issue which restricts the further development of the embryogenesis pathway[8]. On the contrary, the organogenesis pathway exhibits a straightforward regeneration process, facilitating the acquisition of regenerated plants, and effectively preserving maternal genetics[9]. Gollop et al. had presented evidence supporting the stable transformation of non-embryonic callus (NEC) of grapevine[10]. As a result, this approach can be considered appropriate for efficient propagation or genetic transformation, leading to higher yields with reduced production costs[6].

This study utilized the exocarp to induce NEC of different grapevine varieties. Additionally, VyDHN1-overexpressed NEC was successfully obtained, and the study further examined the changes in electrical conductivity, proline content and the quantity of malondialdehyde (MDA) during short-

term cold stress. The outcomes of this investigation may contribute to a better understanding of the mechanisms underlying VyDHN1's response to cold resistance.

2. Materials and Methods

2.1 NEC Induction and Propagation by Exocarps Tissue of Table Grapevine Varieties Zaoxia and 87-1

2.1.1 Explant Sterilization

Berries of table grapevine varieties of Zaoxia and 87-1 were selected at the green berry stage and veraison stage and washed by running water for 4 hours. After that, the berries were disinfected using a mixture of 75% ethanol and 15% sodium hypochlorite then rinsed by sterile water.

2.1.2 NEC Induction and Propagation

Exocarp sliceswere cut and placed backwards on callus induction medium named GP-1(refer to the method of Lai et al.[11]), GP-2(refer to the method of Yamakawa et al.[12]), and GP-3(refer to the method of Bru et al.[13]). The slices were cultured under dark conditions at 27°C for 1 to 2 months.

2.2 Genetic Transformation of Grapevine Callus

2.2.1 Activation of Transformed Bacterial Solution

Plant overexpression vector of VyDHN1 fused with GFP was constructed, i.e. pCAMBIA-2300-35S-VyDHN1-eGFP, and were transferred into the agrobacterium strain GV3101. The transgenic agrobacterium strain was cultured in LB medium supplemented with kanamycin (Kan) and gentamicin (Gent). The method of bacterial solution activation was refered to Metwali et al[14].

2.2.2 Infection, Co-culture, and Screening Culture

The methods were refered to Metwali et al.[14].Two categories of screening methods of NEC transformation were conducted in this study. (a) The NEC clusters after co cultivation were inoculated onto GP-3 delay medium, supplemented with 1000 mg/L Tmt, for a duration of 7 days. (b) The infected NEC clusters were directly inoculated onto GP-3 selectable medium without delay culture.

2.2.3 Fluorescence Verification and Subculture of Transgenic NEC

LUYOR-3410 high-intensity ultraviolet lamp (360~370nm) within the ultra-clean workbench was utilized to irradiate the infected callus. The genetic transformed NEC with GFP signal would be used for further determination the physiological index under cold stress.

2.3 Determination of Physiological Indicators of Cold Stress Callus

Wild type callus (WT) and callus stably over expressed VyDHN1::GFP was subjected to 0°C for 48 hours, while the control group was cultured at 25°C instead. The conductivity measurement method was based on Xu et al[15]. The MDA content was measured using the 2-Thiobarbituric acid method. The method for determining proline content was based on Xu et al.[16].

2.4 Data Analysis

All experiments were repeated three times. The data was analyzed using SPSS 21.0 software (IBM, Chicago, USA) with one-way analysis of variance (One-way ANOVA) and Duncan's multiple comparison test.

3. Results

3.1 Callus Induction and Propagation

Explants at different development stages have the potential impact on callus induction. The results were shown in Figure 1A. Callus induced from the exocarps at the green berry stage of both table

grape varieties exhibited a lightly yellow color, bulky and moist shape. However, callus induced from the exocarps at the veraison stage showed a browning color. Additionally, the rate of callus initiation was significantly lower at veraison. Visual observation of the callus appearance revealed no significant difference between the varieties.

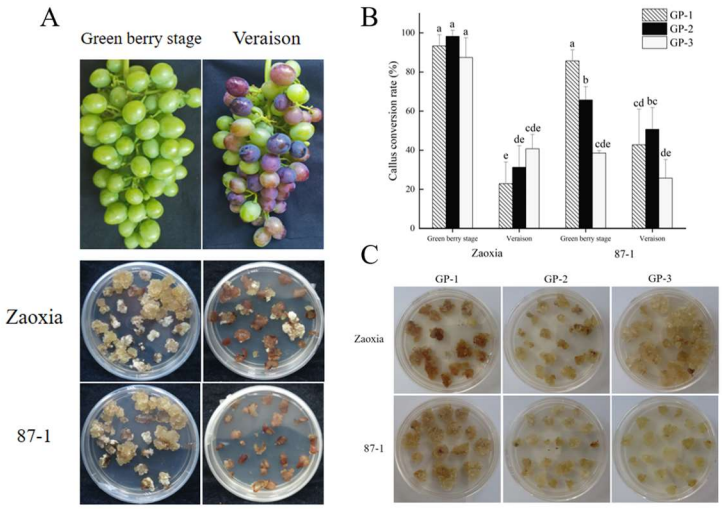

Figure 1. Phenotype (A) and induction rate on three different induction media (B) of non-embryonic callus (NEC) induced by exocarp of berries of Vitis vinifera 'Zaoxia' and '87-1' at green berry and veraison stage and phenotypes(C) of callus on three media GP-1, GP-2 and GP-3 for 4 weeks. Each value is the average of three replicates; error bars are standard deviations; bars with the differen t letters are significantly different at p = 0.05 according to Duncan's multiple range test.

According to the statistics on the induction of NEC from exocarps rate (Figure 1B), it was observed that the NEC initiation rate of all groups exceeded 20%. Moreover, the induction rate was noticeably higher for Zaoxia than for 87-1. Additionally, it was obviously that the NEC induction rate was greater for the explants of the green berry stage for both varieties compared to that of veraison stage. The NEC induction rate for the green berry stage was the higher, ranging from 87.38% to 98.25%, which was approximately 2.4 to 3.8 times higher than that in the veraison stage. In comparison to 87-1, the explant stage had a more significant impact on callus initiation for Zaoxia. In terms of the results for the induction affected by culture media, it was significant that the induction rate of GP-1 in the green berry stage group was consistently high, surpassing 85%. However, the induction rate of GP-2 and GP-3 in counterpart was lower. Nevertheless, it should be noted that there was no notable difference for the induction effects influenced by the induction media for the explants at veraison stage.

As for the expanding propagation of callus, which was shown in Figure 1C, the callus of the two varieties cultured on GP-1 exhibited a gradual browning effect, along with a hardened tissue texture. Similarly, the callus on GP-2 also displayed partial browning. Although the degree of browning was more mild, the growth rate was lower. On the contrary, the callus on GP-3 exhibited the least browning, with a softer and better dispersive texture, indicating better growth performance. Previous studies have demonstrated that callus exhibiting a low degree of browning and a well-dispersed appearance holds advantages for subsequent genetic transformation research[17].

In conclusion, to establishment the NEC regeneration system by exocarp explants of two table grapevines, Zaoxia and 87-1, it was better to select the explants at the green berry stage and induct on medium GP-1, when the callus came out, GP-3 was the most suitable medium for the callus propagation.

3.2 Genetic Transformation of NEC

The green fluorescent protein (GFP) signal of VyDHN1::GFP protein was observed in the third week of the screening culture of Zaoxia (Figure 2A). After a week of delayed culture, a portion of the infected callus gradually turned brown and underwent necrosis. Then the regenerated callus started to emerge with a soft and pale yellow texture after grown on the screening medium GP-3 supplemented with Kan. The newly growth of the positive callus showed GFP signal was strong, and the positive rate was exceeding 50% of the whole infected callus masses. In contrast, the callus that did not undergo delayed phase exhibited severe necrosis, characterized by an overall hard texture and dark browning. The positive rate of fluorescent signals was significantly lower, accounting for only 29.4% which was half as much as that of delayed screening (Figure 2A and 2B). Delayed treatment reduced the browning effect of callus and enhanced the rate of genetic transformation. The screening methods employed did have an important impact on the efficiency of transformation.

Moreover, the efficiency of genetic transformation was significantly influenced by the variety as well. In this experiment, the GFP only be found in the callus of Zaoxia, the 87-1 was failed to genetic modification (Figure 2B).

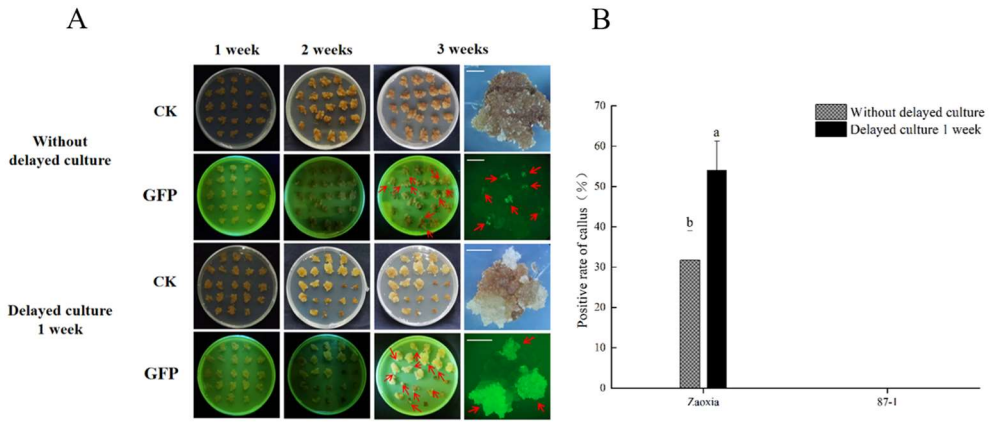

Figure 2. Fluorescence observation of non-embryonic callus of Vitis vinifera 'Zaoxia' after genetic transformation of VyDHN1::GFP at screening culture stage (A) and Positive rate of non-embryonic callus of Vitis vinifera 'Zaoxia' and '87-1' (B). CK: The irradiation light source is white light. GFP: The irradiation light source is LUYOR-3410 high-intensity ultraviolet lamp (360~370nm). Arrows indicated the transgenic callus with GFP signal; scale bar = 2 mm.

3.3 Cell Membrane Relative Permeability Analysis

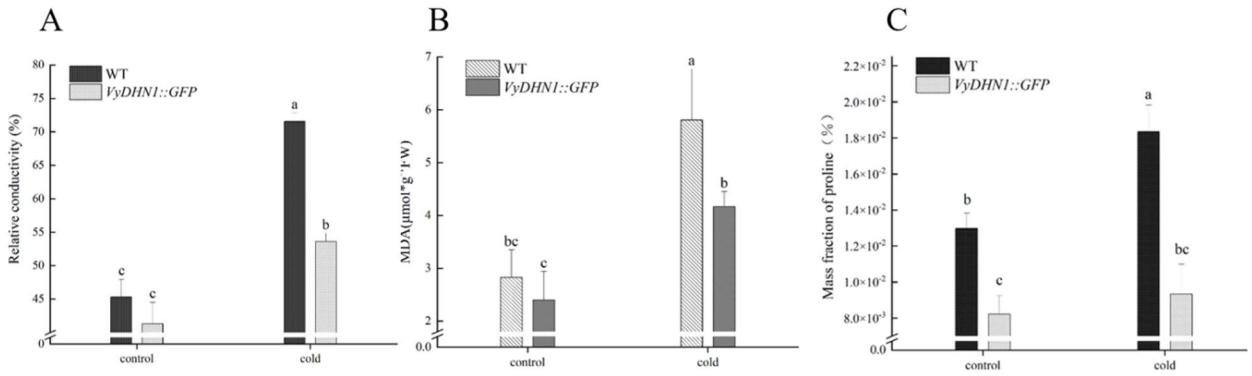

Figure 3. Relative conductivity measurement (A) and MAD (B) and proline mass fraction (C) of WT and overexpressing NEC of 'Zaoxia' at Control (25℃) and Cold (0℃) environment for 48h. Each value is the average of three replicates; error bars are standard deviations; bars with the different letters are significantly different at p = 0.05 according to Duncan's multiple range test.

After subjecting the callus overexpressed VyDHN1::GFP to a low-temperature treatment of 0°C for 48 hours, there was a significant increase in the relative permeability of the Zaoxia NEC cell membrane compared to that of the control group. However, the increase in wild type (WT) (36.68%) was particularly higher after cold treatment, being 1.6 times higher than that of the VyDHN1-overexpressing callus (22.93%) in the cold experimental group.

3.4 Determination of MDA Content

Plant cells experience damage under low temperature stress, leading to the accumulation of reactive oxygen species that induce membrane lipid peroxidation[18]. To assess the extent of membrane system damage and cold stress resistance, the levels of MDA, which is the final product of peroxidation, were indirectly measured after cold treatment. The findings were illustrated in Figure 3B. At room temperature of 25℃, the VyDHN1::GFP-overexpressing NEC of Zaoxia did not yield a substantial alteration in the MDA level. However, following a 48-hour exposure to low temperature of 0℃, the transgenic callus showed a significant increase (73.54%) in MDA content. Additionally, the growth rate of MDA in the wild control group was notably higher (105.86%) than in the VyDHN1 overexpressing group, with the former being 1.69 times greater than the latter.

3.5 Determination of Proline Content

The proline content in plants typically remains low but increases in response to stress[19]. Under normal temperature, the proline content of callus overexpressed VyDHN1::GFP was lower than that of the wild-type. However, after cold stress treatment, the free proline in WT callus increased significantly, about 1.41 times higher compared to the control, whereas the genetic modificatied callus were not shown a significant increase of 13.64% compared to pre-stress levels (Figure 3C).

4. Discussion

Selecting appropriate explants for callus induction is crucial for successful genetic transformation. There are significant variations in differentiation ability among different plant varieties, tissues, and organs. Typically, it is recommended to choose the young parts of the plant during the early or peak growth period when the explants exhibit vigorous division and robust regenerative capabilities[20]. This finding was consistent with our research. As shown in Figure 1A and Figure 1B, the callus induced by the exocarps of both table grapes at the green berry stage showed better callus morphology and initiation rate compared to the veraison stage.

Following the introduction of the foreign gene into the recipient cell, there was a delay in its expression and integration. Consequently, it was advisable to subject the callus to a detoxification culture on a delay medium for a suitable period. This practice served to mitigate the toxic impact of agrobacterium on the callus and enhanced transformation by capitalizing on the nourishing properties of non-transformed cells[21]. This finding was consistent with our conclusion, as depicted in Figure 2. After one week of delayed culture, there was a significant increase in the positive rate of Zaoxia callus. Additionally, there was an increase in the proportion of cells overexpressing VyDHN1::GFP and a decrease in the degree of browning in regenerated cells (Figure 2A and Figure 2B). However, the ability of grapevine callus transformation was constrained by the genotype, resulting in significant variations among different genotypes[20, 24]. In this study, only Zaoxia genotype exhibited successful gene modification and obtained overexpressing VyDHN1 callus masses (Figure 2B).

When plants were exposed to adverse stress, the cell membrane's structure and function were significantly impacted. MDA is a product of membrane lipid peroxidation on cell biological membranes, which could bind to enzymes and proteins on the membrane structure and destroy its structure[23]. The severity of damage to the plant is directly related to the levels of MDA content and relative conductivity. Higher MDA content and conductivity indicate more extensive damage to the plant[18]. Figure 3A and 3B collectively demonstrated that transgenic callus exhibited less damage

compared to wild type cells in cold stress, which showed better protection of callus cell membranes under cold environment and higher resistance to cold when VyDHN1::GFP was overexpressed.

Proline plays a key role in stress resistance. Firstly, it acts as an osmotic regulator maintaining the balance of osmotic pressure between the protoplasm and the external environment to prevent water loss. Secondly, it binds to proteins, it enhances protein hydration and reduces the aggregation of soluble proteins, thereby safeguarding the structural and functional integrity of these biological macromolecules[31, 32]. It is worth mentioning that the accumulation of proline under adverse conditions may not only have adaptive significance but also could indicate damage to cell structure and function, serving as an injury response[26]. Under stress, dehydrin molecules interact with phospholipid molecules in the cell membrane by forming hydrogen bonds with hydroxyl groups, displacing water molecules and helping to preserve the stability of the membrane lipids, mimicking a non-dehydrated state[5, 28]. Dehydrin also functions as a molecular chaperone for important enzymes, providing protection. According to Hincha and Thalhammer, dehydrin is believed to maintain enzyme activity through two possible mechanisms: (1) By utilizing its irregular structure to create a physical barrier that prevents protein aggregation and denaturation[28]. (2) By preventing protein misfolding in order to preserve enzyme activity[28]. Figure 8 showed that the level of proline accumulation in VyDHN1-overexpressing callus after exposure to cold stress was significantly lower. This decrease may be attributed to the role of VyDHN1 in delaying cellular damage processes under short-term stress conditions, leading to an inhibition of the proline regulatory pathway triggered by signaling factors and ultimately resulting in reduced accumulation of free proline.

5. Conclusion

In summary, the induction and propagation of the callus from the exocarp of Zaoxia and 87-1 were established. The exocarp explants at green berry was more efficient for callus induction. Additionally, the best propagation effect of the callus was observed on the GP-3 medium. Furthermore, the genetic transformation of VyDHN1 in Zaoxia callus was successfully achieved, and positive callus overexpressing VyDHN1::GFP were observed after three weeks of screening, which confirming that transformation rate of grapevine callus could be enhanced by delayed culture. Moreover, the overexpression of VyDHN1 could enhance the cold resistance of grapevine by mitigating damage to cell structure in the experiment.

Acknowledgments

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