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Enhancing grapevine transformation and regeneration: A novel approach using developmental regulators and BeYDV-mediated expression

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Abstract. Grapevine (*Vitis vinifera* L.) is a challenging plant species to transform and regenerate due to its complex genome and biological characteristics. This limits the development of cisgenic and gene-edited varieties. One hurdle is selecting the best starting tissue for the transformation process, much like isolating suitable tissue for protoplasts. One promising method involves delivering CRISPR/Cas components to protoplasts isolated from embryogenic calli, which are then induced to regenerate. However, this process is inefficient, time-consuming, and only applicable to a few genotypes. To enhance grapevine regeneration efficiency, the expression of developmental and plant growth regulators shows promise in escaping the recalcitrance encountered in traditional tissue culture methods. A strategy based on the *Bean yellow dwarf virus* (BeYDV) allows for the temporary expression of regulators while minimizing the risk of obtaining transgenic plants. Additionally, the high copy number replication of BeYDV allows for high CRISPR/CAS levels, thereby improving editing. The goal of this study is to evaluate the effects of individual or combined developmental regulators' expression on grapevine embryogenic tissues. We conducted preliminary assays using BeYDV-derived vectors for luciferase reporter gene expression to optimize delivery efficiencies. Assays were performed on 'Chardonnay' calli and protoplasts using both agrobacterium-mediated transformation and protoplast transfection approaches. The present study seeks to enhance the transformation protocols and regeneration processes, with the ultimate aim of realizing the full potential of editing technologies in grapevine.

1. Introduction

Grapevine (*Vitis vinifera* L.) is an essential crop within the global agricultural economy, recognized for its significance not only in wine production but also in the table grape and raisin industries. Despite its economic importance, the genetic enhancement of grapevine remains a huge challenge, primarily due to its recalcitrant nature in tissue culture and transformation processes. These challenges are a crucial bottleneck in developing new, improved cultivars. The difficulty of grapevine transformation can be attributed to its complex genome, which complicates efforts and results in inconsistent success rates across different cultivars [1].

The emergence of New Breeding Techniques (NBTs), particularly CRISPR/Cas-mediated gene editing, has revolutionized the potential for precise genetic modifications in grapevine [2]. Unlike traditional cross-breeding, NBTs allow for the precise alteration of specific traits while preserving the other inherent characteristics of the variety by selectively adding or modifying only target genes. These techniques present unparalleled opportunities for enhancing desirable traits such as disease resistance, abiotic stress tolerance, and fruit quality [3], [4]. However, despite the potential of NBTs, their application in grapevine is often hindered by the species' inherent recalcitrance to both transformation and regeneration [5]. The efficiency of these processes is closely linked to the type of tissue used, with embryogenic calli and protoplasts being favored [6], [7]. Nonetheless, even with these tissues, regeneration efficiency remains low, making the overall process time-consuming and frequently genotype-dependent.

The plasticity and totipotency of plant cells, allow them to regenerate from various tissues in response to stimuli like wounding and hormones. Auxin and cytokinin, two key plant hormones, are crucial in determining the developmental switch and organ regeneration [8]. Despite

their widespread use, efficient regeneration systems are still lacking for many crop species. Recent advancements in plant molecular biology have revealed that the pluripotency of plant somatic cells is controlled by a complex network of developmental regulators (DRs). These regulators are crucial for callus formation and plant regeneration, with mutations in these genes leading to reduced regeneration efficiency [9], [10]. DRs play crucial roles in maintaining meristem identity and promoting somatic embryogenesis [11]. Overexpression of these developmental regulatory genes has been shown to significantly boost the production of transgenic and gene-edited plants. In monocotyledons, combinations such as ZmWus2 and Bbm have been effective in promoting somatic embryo formation [12], [13], while in eudicotyledons, ZmWUS2 combined with STM or ipt induces the development of new meristems [14]. Additionally, wound-responsive DRs, such as PLETHORA (PLT5), contribute to meristem formation, thereby enhancing the efficiency and scalability of plant transformation processes [15].

Further advancing these strategies, the development and optimization of a synthetic gene activator known as dCas9-TV represents significant progress. This system introduces a self-amplification loop to enhance gene activation in plants. Notably, dCas9-TV enables targeted gene activation from native genomic loci, providing a powerful alternative to traditional gene overexpression techniques that often rely on the CaMV 35S promoter, which can be prone to silencing. In rice plants, dCas9-TV has been demonstrated to achieve multiplex gene activation, significantly boosting gene expression levels—reaching up to 40,000-fold in some cases. This robust activation persists across generations, offering a stable and scalable approach to gene editing in plants [16], [17].

In grapevine, the application of DRs offers a promising strategy to enhance tissue regeneration and facilitate genetic transformation. However, the stable integration of DRs into the grapevine genome raises concerns about generating transgenic plants, which may encounter regulatory obstacles and public opposition [5], [18]. To avoid these risks, transient expression systems using virus-based vectors, such as those derived from the *Bean yellow dwarf virus* (BeYDV), have been developed. These systems enable the temporary expression of DRs, thus reducing the likelihood of creating transgenic plants. BeYDV-based vectors are particularly beneficial due to their high replication rate, which allows for the accumulation of substantial levels of CRISPR/Cas components, further improving the efficiency of genome editing in recalcitrant species like grapevine [14], [16].

This study investigates the use of BeYDV-mediated transient expression of DRs to enhance transformation and regeneration processes in grapevine. By optimizing the delivery of these regulators to embryogenic tissues, we aim to overcome the recalcitrance of grapevine and improve the efficiency of CRISPR/Cas-mediated gene editing. Preliminary assays on 'Chardonnay' calli using Agrobacterium-mediated transformation approaches seek to refine transformation protocols and regeneration processes, maximizing the potential of editing technologies in grapevine. Additionally, we are trying to explore the potential for further improvement by activating grapevine-specific DRs through engineering dCas9-TV, aiming to enhance transformation efficiency and overall growth outcomes.

2. Materials and methods

2.1. DNA vectors

pMM131, pMM134, pMM113, pMM114, pMM146, and pMM135 vectors were purchased from Addgene and introduced in Agrobacterium tumefaciens GV3101 strain competent cells. While, dCas9-TV was excised from pCAMBIA-dCas9-TV-1-only vector kindly provided by Dr. Jian-Feng Li and its molecular engineering is ongoing.

2.2. Agrobacterium-mediated transformation

Agrobacterium-mediated transformation and embryo selection have been performed as reported by Bouquet et al. [19] with minor modifications. Briefly, the GV3101 agrobacterium strain containing the vector of interest was incubated overnight at 28°C in LB medium; the optical density was adjusted to 0.4. The bacterial suspension was mixed with Chardonnay calli, incubated in TB medium plus acetosyringone, and transferred to GS1CA medium in the dark at 22°C. After 2 days of co-cultivation, the calli were washed with TB plus timentin, fragmented, and placed on GS1CA medium with timentin for 3 weeks. Subcultures were performed every 3 weeks, and then developed embryos were transferred to FIII medium under light.

3. Results

3.1. Choice of vectors for transient expression

BeYDV-derived vectors (pMM131, pMM134, pMM113, pMM114, pMM146, and pMM135) were selected for their high replication rates and and capacity to induce transient expression of DRs in grapevine tissues. Vectors were selected from those used by Maher et al. [14], and were purchased from AddGene. These vectors contain key developmental regulators (ipt, STM, WUS, BBM) and a luciferase reporter gene to monitor expression. The highest luciferase activity was exhibited by the ipt vector (pMM134), indicating successful transformation and robust transgene expression (Figure 1).

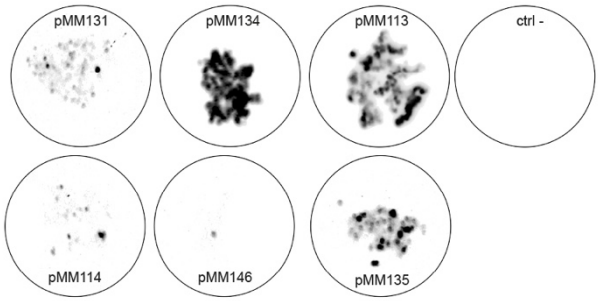

Figure 1. Bioluminescence detection. Chemidoc bioluminescence detection of Chardonnay calli transformed via agroinfiltration with developmental regulators contained in a BeYDV vector and luciferase reporter gene.

3.2. Transformation and plantula regeneration

The transformation of 'Chardonnay' calli using Agrobacterium and these vectors resulted in varying expression levels, with the ipt vector (pMM134) achieving the highest (FIgure 1). Luciferase activity was observed to persist for up to a month, demonstrating the effectiveness of the BeYDV system in maintaining expression long enough to influence regeneration. The developmental progress of both transformed and control calli was tracked over a four-month period (Figure 2).

Figure 2. Four-month tracking of transformed calli. Development of Chardonnay embryos into plantula in the negative control and in ipt-stimulated calli.

Significant shoot formation was observed in the ipt-transformed calli, with a 75% regeneration rate compared to 28.6% in controls (Figure 3). No enhancement in regeneration was observed with other developmental regulators. As a preliminary test, among the regenerated plantlets, leaves from two ipt-treated and one from the control have been tested through end-point PCR to assess if there was foreign DNA integration. All the tested leaves were PCR-negative. Real-time monitoring of luciferase expression will provide further insights into the temporal dynamics of transgene activity.

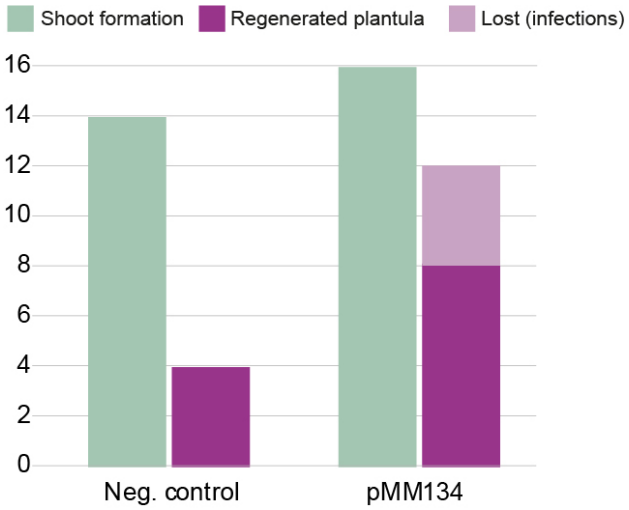

Figure 3. Regeneration efficiency. Histogram showing the regenerated plantulas from the pMM134 transformed calli compared with those regenerated from the negative control.

3.3. Transformation efficiency via dCas9-TV activation of grapevine DRs

The dCas9-TV system was explored for activating grapevine-specific DRs, with a focus on the pCAMBIA-dCas9-TV-1-only vector. Preliminary results have been promising, showing the potential to enhance transformation efficiency by targeting native grapevine genes without the integration of foreign DNA.

4. Discussion and Conclusion

Grapevine (*Vitis vinifera* L.) is a crucial crop in the global agricultural economy, but its genetic improvement is challenging due to its recalcitrant nature in tissue culture and transformation processes [1]. The introduction of NBTs like CRISPR/Cas has revolutionized precise genetic modifications [1], yet grapevine's recalcitrance to transformation and regeneration remains a significant hurdle [5].

Here, we explore innovative strategies to overcome these challenges by using DRs delivered via a BeYDV-mediated system. Our findings highlight that the BeYDV-based transient expression system, particularly with the IPT regulator, significantly enhances regeneration in 'Chardonnay' calli, achieving a 75% regeneration rate compared to 28.6% in controls (Figure 3). This underscores the crucial role of specific DRs, especially in cytokinin biosynthesis, for successful regeneration. The transient expression system is advantageous for reducing the risks of stable foreign DNA integration, aligning with the growing emphasis on enhancing plant traits without transgenesis. Moreover, the preliminary use of the dCas9-TV system for activating grapevine-specific DRs also shows promise for future research, offering the potential for targeted activation of native genes without the drawbacks of transgenic methods.

Overall, BeYDV-mediated expression and the employments of dCas9-TV present a promising framework for advancing gene editing in grapevine.

Further refinement and broader application of these techniques across various grapevine genotypes could significantly advance viticulture by developing new varieties with improved traits. Future studies should focus on refining these strategies, exploring additional DR combinations, and assessing long-term effects on regeneration efficiency.

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