REFEREED MANUSCRIPT

Proc. Fla. State Hort. Soc. 124:13–17. 2011.

Genetic Transformation for Overexpression of Flavonoid Compounds in *Muscadinia* Grape Cell Cultures

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ADDITIONAL INDEX WORDS. grape, *Vitis,* muscadine, flavonoids, somatic embryogenesis, anthocyanin, *MYB*

Among the unique changes that take place during grape berry development, those affecting the flavonoid pathway have triggered a number of investigations for over a decade. Anthocyanins, which are expressed in red and black grape cultivars and responsible for the color of red wine, are biosynthesized via the flavonoid biosynthetic pathway. The *MYB* gene is known to encode a transcription factor that induces the anthocyanin biosynthesis in grapes. In this article, we describe the transformation of the somatic embryos of 'Supreme' and 'Darlene' varieties of muscadine, with targeted constitutive ectopic expression of *MYB* gene in grape somatic embryogenic callus. The ectopic expression of *MYB* triggered de novo production and storage of anthocyanins in all transgenic cells including the hypocotyl, leading to a very intense red coloration. The ectopic red pigments were as a result of the accumulation of anthocyanins in the tissue. Molecular analysis using gene specific primers confirmed the proper integration and overexpression of the *MYB* gene. Our results strongly support the previous studies that *MYB* genes are involved in the regulation of anthocyanin biosynthesis in the grape via expression of the UFGT gene.

The common muscadine grape is native to the southeastern United States and has been cultivated for more than 400 years. The discovery of high concentrations of antioxidant compounds in muscadine berry, juice, and wine has brought increased attention to muscadine grape, not only as an important alternative cash value crop for the southeastern United States, but as a new healthy food as well (Colova et al*.,* 2007). Muscadine grapes are considered one of the most important *Vitis* species because of their containment of several unique flavonoid compounds that have beneficial nutraceutical properties. They are the only grapes containing ellagic acid, and possess one of the highest antioxidant levels among fruits (Samuelian et al., 2009). With the rising interest in attaining wellness through diet, consumer interest in the relationship between diet and health is rapidly expanding. The U.S. interest in functional foods is also fueled by different factors such as the advances in science and technology, increasing health care costs, changes in food laws affecting label and product claims, and an aging population (International Food Information Council Foundation, 2007).

Scientists have envisioned the power of genetic engineering to enhance nutritional and other properties of foods for consumer benefits. Even though the first generations of agricultural biotechnology products to be commercialized were more geared toward transgenes that targeted modifications that made insect, virus, and weed control easier or more efficient (Pew Initiative on Food and Biotechnology, 2007), the new generation has promoted technological advances in learning more about the genes and biochemical pathways controlling the attributes that could offer more direct consumer benefits.

Most grapevine cultivars can be divided into two groups, red and white, which is based on the expression of anthocyanin in the berry skin (Walker et al., 2006). The berry color locus comprises two very similar genes, *VvMYBA1* and *VvMYBA2,* and either gene can regulate color in the grape berry (Walker et al., 2006). Therefore, *MYB*-related transcription factor genes such as *vVMYB* can regulate anthocyanin biosynthesis (Kobayashi et al., 2002). Anthocyanins are red or purple colored secondary metabolites that are present in the leaves, stems, flowers, and fruits of many plants. Their accumulations are mostly responsible for pigmentation in red and black grapes, which is essential in wine making, and they are localized in the berry skin. Genes that affect early stages in the biosynthesis of anthocyanins often result in colorless phenotypes; however, if a mutation exists later in the pathway it may only affect the types of anthocyanins produced (Angelakis-Roubelakis, 2001).

The *MYB* gene family encodes nuclear proteins that function as transcriptional transactivators (Yousef et al., 2004). Transactivation by the *MYB* gene family varies considerably depending on cell type and promoter context, suggesting a dependence on interaction with other cell type specific co-factors (Li et al., 2006). *MYB* is a marker gene that aids in overexpression of bioflavonoid compounds. It has been proved to be a valuable marker for transformation due to its ability to regulate anthocyanin synthesis effectively (Walker et al., 2006).

In this study, we used *Agrobacterium tumefaciens* harboring the *MYB* gene to transform embryogenic callus of 'Darlene' and

Acknowledgments. This research was supported by USDA/NIFA/AFRI Plant Biochemistry Program Grant # 2009-03127, and SUS Fellowship Program–FAMU School of Graduate Studies. We extend our appreciation to all members of the FAMU/CESTA Center for Viticulture & Small Fruit Research.

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'Supreme', with the purpose of producing transgenic embryos that overexpress the *MYB* gene. This will eventually shift the metabolic flux of flavonoids in transgenic lines. This approach will also allow us to assess the nutraceutical value of commercial North American grape varieties, and to contribute knowledge about genomic mechanisms affecting the production of flavonoid compounds in grape. In addition, it proves to be a feasible technology for using genomic sequences to improve nutritional and health benefits of the native grapes.

Materials and Methods

Plant materials. In vitro callus derived from petioles of white and red North American native grapes *Muscadinia* 'Darlene' and 'Supreme' (Fig. 1) were used in this study. Somatic embryogenic callus from these cultivars was obtained from our lab and maintained in a CP1 and MS solid medium as described by Krastanova et al. (2000) and Perl et al. (2004) with slight modifications. The cells were grown in the growth chamber at 28 °C in the dark. Figure 1 shows the clusters at the physiological maturity stage of the two varieties that were used to initiate the in vitro embryogenic cell lines.

Plasmid constructs. The plasmid construct that was used was generously provided by Dr. Avihai Perl from the Department of Fruit Tree Breeding, ARO The Volcani Center, Israel.

Tissue culture medium. Culture medium for growing *A. tumefaciens* was prepared as defined by Krastanova et al. (2000) and Perl et al. (2004). Two kinds of media, i.e., modified Murashige and Skoog (MS) and modified Chee & Pool (CP1) were used for maintaining the somatic embryogenic culture lines. Subculturing of embryogenic calli was carried out on solid, full-strength MS medium containing 20 g/L sucrose, 0.2 mg·L^{-1} 6-benzyl-aminopurine (BAP) and 1.1 mg·L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (induction medium) in the dark at 28 °C (Krastanova et al., 2000). The pH was adjusted to 5.7 and Noble agar was added to a final concentration of 7.2 g·L^{-1}. Cultures were maintained by transferring clumps of embryos to induction medium at 20-d intervals for calli multiplication. After 30–45 d, only secondary calli were transferred to half-strength MS medium supplemented with 3.6 mL·L^{-1} glycerol for embryo differentiation. After 3 weeks, cultures were transferred to solid MS medium containing 1 g/L casein hydrolysate and 3.6 mL·L^{-1} glycerol for embryo elongation. For the regeneration media, the protocol designed by Colova et al. (2007) was used.

Agrobacterium-mediated transformation. Two transformation protocols were used in this study in order to find the most efficient method. Method I was based on the protocol of Krastanova et al. (1993, 1995, 2000, 2010): Two sterilized Erlenmeyer flasks each containing 25 mL of YEP media with 25 µL of tetracycline (12.5 mg·mL^{-1}), rifampicin (30 mg·mL^{-1}), and kanamycin (50 mg·mL^{-1}) were prepared. Two single colonies of *A. tumefaciens* strain EHA105 harboring a *MYB* plasmid were obtained, placed in individual flasks, and grown in darkness in an incubator/shaker at 28 °C at 120 rpm for 24 h. After the bacteria had developed, 15 mL was taken from each flask, placed in a 50-mL conical tube, and centrifuged for 10 min at 3000 rpm. The supernatant was discarded and the pellet was re-suspended with one-half MS liquid media supplemented with 100 µM acetosyringone and used for co-culture with *A. tumefaciens*. The OD was adjusted to 0.525–0.6. Embryogenic calli from 'Darlene' and 'Supreme' were incubated in petri dishes containing *A. tumefaciens* suspension for 20 min blotted on sterile paper. , The embryogenic calli were split into two different culture media, MS and CP1, containing 100 mg·L^{-1} acetosyringone. The plates were placed in the incubator at 25 °C for 48 h in the dark. After co-culture the embryogenic calli were transferred to MS supplemented with 500 mg/L cefotaxime, 50 mg·L^{-1} kanamycin and maintained at 25 °C in the dark for selection. Non-transformed embryogenic calli were grown in the same medium with or without kanamycin to verify the efficiency of the antibiotic selection. Method II was done following a protocol from Perl et al. (2004) with slight modifications.

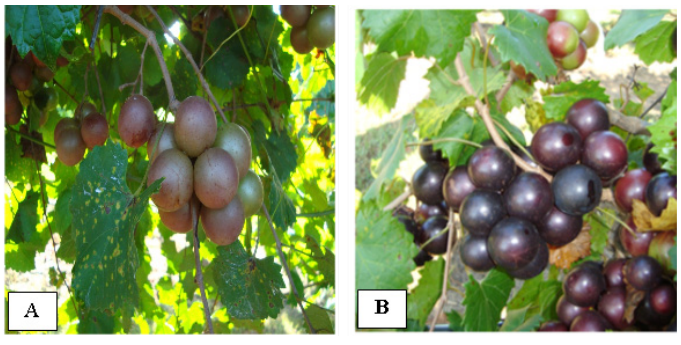

Fig. 1. Commercial muscadine grape varieties used in the study: (**A**) 'Darlene', (**B**) 'Supreme'.

Molecular analysis of transformed material

RNA extraction. Total RNA was isolated from 100 mg of somatic embryos from transformed (reddish color) and non-transformed (control) embryos using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Purified RNA was treated with RNase-free DNase 1.

RNA gel electrophoresis. To evaluate RNA quality, it was necessary to perform formaldehyde gel electrophoresis. For 100 mL of gel mixture, 10 mL of 10× MOPS stock solution was mixed with 1.2 g of agarose (makes a 1.2% gel) and 90 mL DEPC H_2O. The gel mixture was heated in a microwave to dissolve agarose and then 1.8 mL of 37% formaldehyde was added (under the hood). The gel apparatus (including the gel tray and comb) were treated with RNase Away and rinsed out with distilled water. Once solidified, the gel was placed in the gel tray, and 250 mL of running buffer (25 mL 10× MOPS, 9 mL 37% formaldehyde, and 216 mL H_2O) was added to the tray. To load the gel, it was critical to add at least 1 µg of RNA sample per well. RNA samples were thawed, an aliquot was removed and the remaining sample was re-frozen immediately in –20°. A 1:1:1 ratio of loading buffer: formamide: RNA sample was mixed together, heated at 100 °C (boiling water bath) for 3 min and centrifuged briefly before loading on gel. The gel was run at 70 V for 1–1.5 h.

cDNA synthesis. Total RNA isolated from somatic cells of 'Darlene' and 'Supreme' were used in primary gene expression profiling. The SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize cDNA in a 20 µL reaction containing 1 µg of DNase I-treated total RNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (DDT), 0.5 µg oligo (dT), 0.5 mM each of dATP, dGTP, dCTP, and dTTP, and 200 U SuperScript II Reverse Transcriptase. RNA, dNTPs, and oligo (dT) were mixed first, heated to 65 °C for 5 min, and placed on ice until addition of the remaining reaction components. The reaction was incubated at 50 °C for 50 min, and terminated by heat inactivation at 85 °C for 5 min. The cDNA product was treated with 1 µL of Rnase H

(Invitrogen) for 20 min at 37 °C. An identical reaction without the reverse transcriptase was performed to verify the absence of genomic DNA (no-RT control).

Real time PCR (RT-PCR) analysis. Primers used in this study were designed based on Samuelian et al. (2009) and Kobayashi et al. (2002) and were used to amplify partial gene-specific sequences of *MYB*. A list of primer pairs and genes targeted are provided in Table 1. A cDNA from somatic cell lines of transformed and non-transformed 'Darlene' and 'Supreme' were used. The following procedures were used from the BIO-RAD iQ™ SYBR Green Supermix kit for RT-PCR. A master mix was made for each primer pair included the following: 1) 25 µL of iQ SYBR Green Supermix, 2) 1 µL of the forward primer, 3) 1 µL of the reverse primer, 4) 19 µL of sterile water, and 5) 3 µL of cDNA for a total of 50 µL to be placed in each well on the RT-PCR plate. RT-PCR amplification and analysis were performed using iCycler iQ Multicolor Real-Time PCR Detection system (Bio-Rad; Vandesompele et al., 2002). The threshold cycle (C_t) was determined and the assays were optimized based on the concentration of the primer pairs, and the annealing temperature. Expression of constitutive *Actin* and *Ubiquitin*, two housekeeping genes of *Arabidopsis thaliana,* were used to normalize the expression results using gene expression analysis. Differences in gene expression among transformed and non-transformed cells of *Muscadinia* 'Darlene' and 'Supreme' were calculated according to the delta-delta C_t method (Pfaffl, 2001). RT-PCR was performed under the following conditions: an initial denaturating cycle (2 min at 95 °C), followed by 40 cycles constituted by three steps of denaturation, annealing, and polymerization (20 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C). PCR amplification was done in a 25 µL total volume, containing 3 µL of diluted cDNA (duplicate), 0.5 µm of each primer, and 11 µL of 2× iQ SYBR Green Supermix. In each experiment, target and housekeeping genes were amplified in the same plate with a series of diluted cDNA (10^{-5} to 10^{-9} ng) to generate calibration-specific curves. Three independent experiments were performed for each sample. RT-PCR products were analyzed on a 1% of agarose gel in 1× TBE buffer with ethidium bromide staining. *Actin* and *Ubiquitin* primers were used to optimize the RT-PCR conditions. An optimal condition for amplification of *MYB* gene was determined using an annealing temperature (59 to 60 °C) and by testing a range of concentrations to establish optimal concentrations of primers. Amplification was carried out in iCycler iQ® 96-well PCR plates (part number 2239441, Bio-rad) sealed with Microseal® 'B' film (part number MSB1001, Bio-rad). All samples, including the no template negative control were run in triplicates. A sample was considered positive and C_t value was determined after it crossed the minimum threshold level above background fluorescence after base-line subtraction.

Statistical analyses. C_t values from the iCycler iQ Multicolor Real-Time PCR Detection system (Bio-rad) were analyzed. The statistical significance of experimental variation was calculated using Tukey at confidence levels of 95% ($P < 0.05$). A P-value superior to 0.05 indicated that no difference of variation of expression could be deduced.

Table 1. PCR primers used for real-time PCR and the genes targeted as well as expected sizes for amplified fragments.

Gene targeted	Primer name	Sequence of forward (F) and reserve (R) primers	Amplified fragment (bp)
MYB	Vv_10009764	F 5'-GGCTTCACAATTTGTTGGGG-3' F 5'-TGCTGCAGTTTCTTCTGTCC-3'	215
MYB	Vv_10006876	F 5'-AAACTCATGGTGGACTGCAC-3' F 5'- AAGTCGGTTCCTAAGTGCTC-3'	145
Actin	Actin	F 5'-TAGAAGCACTTCCTGTGGAC-3' R 5'-GGAAATCACTGCACTTGCTC-3'	125
Ubiquitin	Ubi-arab	F 5′-ACTCTCACCGGAAAGACCATC-3′ R 5′-TCACGTTGTCAATGGTGTCAG-3′	112

Results and Discussion

Primers used were specific to the targeted gene. Melting curve analysis for each amplicon showed a single peak (data not shown), confirming the specificity of the primers used in RT-PCR for all target genes. A single amplicon showing the DNA fragment of the expected size was shown in the agarose gel electrophoresis with no visible primer-dimer products. All these results indicated that the total RNA and the derived cDNA template were free of contaminating genomic DNA, demonstrating high quality of nucleic acid preparations obtained for gene expression level analyses by RT-PCR.

Verification of housekeeping genes. An appropriate endogenous reference (housekeeping) gene is critical for RT-PCR to normalize errors and sample-to-sample variations. An ideal endogenous reference gene should be species specific and have single or low copy numbers per haploid genome as well as low heterogeneity across genotypes within the species (Baeumler et al., 2006; Zhang and Hu, 2007). In the current study, *Actin* and *Ubiquitin* (encoding actin and ubiquitin-conjugating enzymes, respectively) were selected as endogenous reference genes. Primers were designed to amplify and hybridize to these two houskeeping gene sequences only. To evaluate the stability of expression of housekeeping genes, RNA transcription levels for 'Darlene' and 'Supreme' in vitro transformed and non-transformed callus were measured. *Actin* and *Ubiquitin* showed the highest expression level (having lower C_t values). The C_t values of the housekeeping genes were not significantly different from each other, but the RNA transcription level varied between the housekeeping genes and the *MYB* gene.

Expression of the *MYB* gene in transgenic somatic embryos. Somatic embryos from 'Darlene' and 'Supreme' capable of differentiation were used as target tissues to insert the *MYB* transgene. The *MYB* gene was driven by cauliflower mosaic virus 35S (CaMV 35S) promoter and immobilized in *Agrobacterium* strain EHA 105. Stable independent lines of embryogenic callus and hypocotyls were recovered from the callus that was inoculated with the *MYB* gene. The majority of the callus and hypocotyls exhibited an expected phenotype, overexpression of the red color (Figs. 2B, 2D, and 3). The embryos and hypocotyls expressing the *MYB* gene harbored a red coloration that was very noticeable. Non-transformed embryogenic callus and hypocotyls showed the same growth pattern, but exhibited only a white to pale yellow color (Fig. 2 A and C). The cultivars that were investigated, 'Darlene' and 'Supreme', produced somatic embryogenic callus and hypocotyls with the same anthocyanin distribution pattern. Regeneration of well-transformed plants is still ongoing. Using *MYB* specific primers, the expression of the *MYB* transgene was evaluated in several transformed and non-transformed cell lines and hypocotyls to validate the presence of the *MYB* transgene. Strong overexpression of *MYB* in the transformed callus and hypocotyls was observed (Fig. 4). In the non-transformed embryogenic callus, the expression of the *MYB* gene was weak.

MYB induced anthocyanin pigmentation in embryogenic

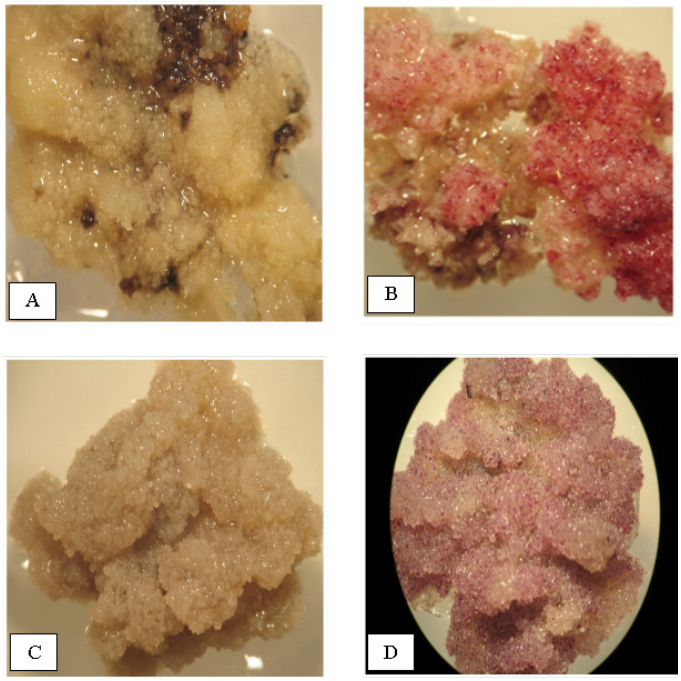

Fig. 2. Embryogenic callus from muscadine grapes, phenotypically expressing the MYB transgene. (**A**) 'Darlene', non-transformed; (**B**) 'Darlene', transformed; (**C**) 'Supreme', non-transformed; and (**D**) 'Supreme', transformed.

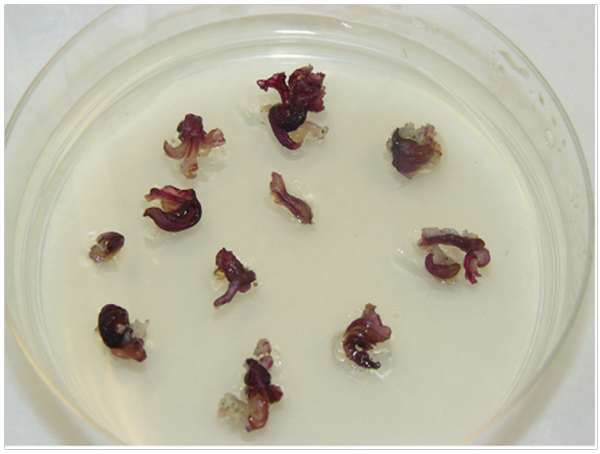

Fig. 3. Transformants in hypocotyl stage (10–12 months) from 'Supreme'. These lines are being regenerated into full plants.

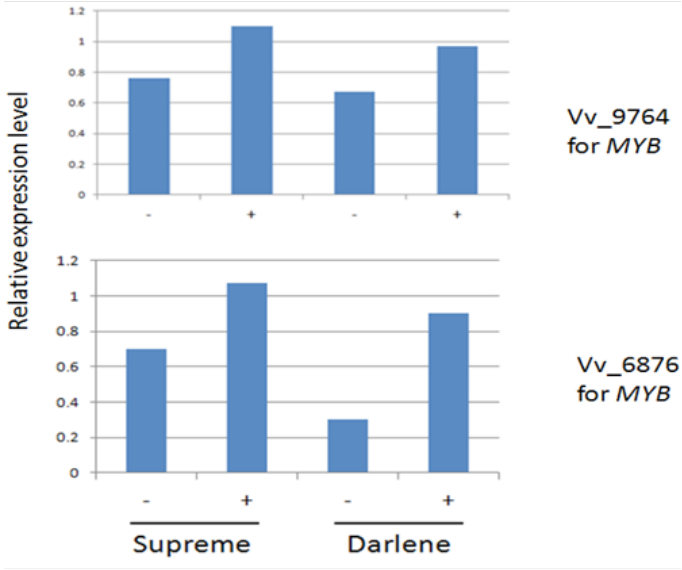

Fig. 4. Real-time expression analysis of the transcripts of MYB in somatic embryos of 'Supreme' and 'Darlene' (control indicated by – and MYB transformed indicated by +). Vv_9764 and Vv_6876 represent the primers that target the MYB gene. All data are the mean (±SD) of three replicates on three independent transformation lines. Expression values were normalized with *Actin* and *Ubiquitin*.

CALLUS. The results presented here suggest that ovexpression of the *MYB* gene induced the anthocyanin pigmentation of the embryogenic calluses, and hypocotyls of muscadine, demostrating the effect of this gene on the induction of anthocyanin pigmentation. Geekiyanage et al. (2007), and Koshita et al. (2008) also reported that *VlmybA2* and *VlmybA1-2* could induce complete red pigmentation in several dicot plants, such as tobacco, *Arabidopsis*, kiwi fruit, tomato, and eggplant. Our study strengthens their research by showing that the same can be applied in embryogenic callus of muscadine grapes. In the non-transgenic lines there was no red pigmentation in the embryogenic calluses and hypocotyls (Fig. 2 A and C) and the mRNA expression of the endogenous *MYB* was weak as compared to the transformed lines, suggesting that the red phenotype was actually due to the activation of the anthocyanin pathway by the *MYB* transgene. Thus, possible autoactivation mechanism of the anthocyanin pathway was ruled out. As stated by Cutanda-Perez et al. (2009), activation tagging in *Arabidopsis* and tomato has led to the identification of the *MYB*-related regulatory gene, *PAP1* and *ANT1*, which share similarities with *MYB*. The overexpression of these *MYB*-type genes in *Arabidopsis* and tomato lead to the formation of an intense purple coloration in the vegetative organs (Borevitz et al., 2000; Mathews et al., 2003; Zuluaga et al., 2008).

Conclusion

Our study suggest that an overexpression of the *MYB* gene in transformed embryogenic callus of muscadine is an indication that regulatory genes can be used to modify secondary metabolic pathways in a predictable manner. Therefore, this process can be explored to shift the metabolic flux in the anthocyanin biosynthetic pathway. Further research is needed to understand how *MYB* coordinates the expression of genes found in the anthocyanin biosynthetic pathway in muscadine grapes.

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