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Large-scale characterization of promoters from grapevine (*Vitis* spp.) using quantitative anthocyanin and GUS assay systems

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ABSTRACT

Successful implementation of cisgenic/intragenic/ingenic technology for crop improvement necessitates a better understanding of the function of native promoters for driving desired gene expression in host plant. Although the genome of grapevine (*Vitis vinifera*) has been determined, efforts to explore promoter resources for the development of cisgenics are still lacking. Particularly, there is a shortage of constitutive promoters for marker and/or target gene expression in this species. In this work, we utilized an anthocyanin-based color histogram analysis method to evaluate quantitatively a large number of promoters for their ability to activate transgene expression. Promoter fragments corresponding to known genes were amplified from various genotypes and used to drive the *VvMybA1* gene of 'Merlot' for anthocyanin production in non-pigmented somatic embryo (SE) explants to infer transcriptional activity. Results revealed that among 15 tested promoters belonging to seven ubiquitin genes, at least three promoters generated constitutive activities reaching up to 100% value of the d35S promoter. In particular, the high activity levels of VvUb6-1 and VvUb7-2 promoters were verified by transient GUS quantitative assay as well as stable anthocyanin expression in sepal and corolla of transgenic tobacco. Variations in promoter activity of different ubiquitin genes in grapevine did not correlate with the presence and sizes of 5′ UTR intron, but seemed to be related positively and negatively to the number of positive *cis*-acting elements and root-specific elements respectively. In addition, several of the 13 promoters derived from a PR1 gene and a PAL gene produced a higher basal activity as compared to previously reported inducible promoters and might be useful for further identification of strong inducible promoters. Our study contributed invaluable information on transcriptional activity of many previously uncharacterized native promoters that could be used for genetic engineering of grapevine.

1. Introduction

In recent years, concerted efforts in genetic manipulation of crop plants via the cisgenic/intragenic/ingenic approach (cisgenics hereafter) have gained an accelerated momentum due to the rapid progress in plant genomics and proteomics. As compared to transgenics technology that primarily relies on the use of foreign genetic materials to modify recipient species, cisgenics technology specifically utilizes reengineered native genes and genetic elements to improve host species. Cisgenic plants are considered to be essentially similar to plants bred through conventional hybridization method. In addition, cisgenic products will require less stringent regulatory scrutiny designed to prevent unintended dissemination of transgenes to the environment while boosting consumer acceptance and confidence [1–4].

Grapevine (*Vitis vinifera* L.) is being grown worldwide with significant economic impact on many producing regions. On the other hand, traditional breeding programs for improving resistance and agronomic performance of this crop are often hampered by many limiting factors, including difficulties to achieve unique enologic quality and wine attributes consumers preferred, long life cycle, self-incompatibility and highly heterologous genetic milieu associated with vegetative reproduction [5]. The advances in the refinement of tissue culture and genetic transformation techniques and the completion of grape genome provided an unparalleled opportunity for introduction of native genes and genetic elements into elite grape varieties for trait improvement without changing other existing characteristics [6–8]. However, efforts are still needed to resolve obstacles to the implementation of such cisgenics technology in grapevine. For the time being, there is a critical shortage of known native promoters that can be readily used to direct desired expression of native genes in engineered grape plants.

Promoter is the single most important genetic element orchestrating gene expression both qualitatively and quantitatively. Over the last three decades, a large number of promoters have been

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Table 1
Chromosome locations and predicted introns of 1 kb ubiquitin gene promoter fragments derived from the 'Pinot Noir' genome.

Promoter	Chromosome[a] (12×)	Promoter			Intron			Gene accession no.
		Start	End	Orientation[b]	5′-Splicing[c]	3′-Splicing	Size (bp)	
VvUb1	4	4271375	4272374	Reverse	509	793	285	N/A
VvUb2	7	4556007	4557006	Sense	637	755	119	GSVIVP00028185001
VvUb3	8	4578661	4579660	Sense	N/A	N/A	N/A	GSVIVP00027151001
VvUb4	8	17077647	17078646	Sense	N/A	N/A	N/A	N/A
VvUb5	16	21450319	21451318	Reverse	N/A	N/A	N/A	AM434449
VvUb6	19	5889905	5890904	Sense	566	832	267	CBI22354
VvUb7	19	5914878	5915877	Sense	569	843	275	N/A

[a] Ubiquitin gene promoters were identified from corresponding chromosomes of the 12× assembly of the 'Pinot Noir' genome.
[b] Orientation of the promoters is shown based on sequence orientations of the published chromosomes.
[c] Intron splicing sites within each 1 kb promoter were identified using NetPlantGene server for predictions of splice sites in *Arabidopsis thaliana* DNA: http://www.cbs.dtu.dk/services/NetPGene/. N/A denotes no matching splicing sites or gene accession hits.

isolated from a wide range of plant species and characterized in great detail. In general, promoters function in plants to support constitutive, inducible, tissue-specific and developmentally regulated expression [9]. In particular, constitutive promoters direct high levels of gene expression in all cell types throughout the entire period of growth and development, and thus provide a broader application in genetic engineering programs [10]. In grapevine, the majority of promoters used in transformation studies were mainly derived from viral origin [11–15]. It has become more urgent to identify and characterize native promoters with constitutive activity and other desired regulatory control to replace viral promoters used in this species.

In this study, we isolated a total of 31 promoters from various genotypes of grapevine and extensively characterized their transcription activity using transient expression of both anthocyanin and GUS reporter genes and associated quantitative analysis methods. We showed for the first time that a number of ubiquitin gene promoters were capable of directing high-level of gene expression comparable to the double enhanced CaMV 35S promoter. Stable transgene expression driven by these native promoters was also determined in transgenic tobacco. These promoters provide an invaluable tool for both promoter development and cisgenic engineering of grapevine.

2. Materials and methods

2.1. Plant culture preparation and transformation

Somatic embryos (SE) (*V. vinifera* cv. 'Thompson Seedless') were initiated from young leaves of in vitro-grown shoot tips and maintained on X6 medium according to Scorza et al. [11]. SE at mid-cotyledonary stage of development were used for transformation analysis. Transformation procedures and culture conditions specifically designed for anthocyanin expression were described previously [16].

Tobacco seeds (*N. tabacum* cv. 'Samsun') were sterilized in 50% bleach solution for 10 min followed by three rinses with sterilized water. Seeds were plated on Petri dishes containing MS medium and allowed to grow under light for one week. Cotyledonary leaves were collected and used for transformation and plant regeneration according to Burrow et al. [17]. Transgenic tobacco plants were established in potted soil in the greenhouse for examination of anthocyanin expression.

2.2. Genomic sequence source and promoter isolation

All promoter sequences were identified by searching available public genomics databases including NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank) that contains the genome of *V. vinifera* 'Pinot Noir' clone ENTAV 115 and Genoscope (http://www.genoscope.cns.fr/cgi-bin/blast_server/projet_ML/blast.pl) with the genome of 'Pinot Noir' clone PN40024. Retrieved DNA sequences from different sources were verified by alignment analysis using the Vector NTI Advance™ software version 10.3.0 (InVitrogen, Carlsbad, CA, USA) prior to use as a template for PCR amplification.

Promoters corresponding to genes encoding ubiquitin (herein named VvUb), a previously described PR1 protein XP_002273416 (named VvPR1) [18], 2S albumin protein (named VvAlb1) [19], cytochrome P450 (named VvGP450), phenylalanine ammonia-lyase (PAL) (named VvPAL1) were tested. Since many ubiquitin genes contain an inconspicuous intron in the 5′-untranslated region (5′-UTR), we used the simple term promoter to describe the specified sequence region upstream from the translation start site of a corresponding ubiquitin gene, which included upstream transcription regulatory sequence and a 5′ UTR region with or without an intron. A total of seven ubiquitin genes identified from the genome of 'Pinot Noir' were used to retrieve promoters from genomic DNA of different varieties after PCR amplification. Chromosome locations, promoter sequence orientations and intron splicing sites of these ubiquitin gene promoters were listed in Table 1.

Phylogenetic analysis and dendrogram reconstruction were carried out using MEGA5 software [20]. The Neighbor-Joining (NJ) method with 2000 replications and a bootstrap value of >70% as significant branch limit was employed [21,22].

2.3. Template DNA isolation and PCR amplification

Total genomic DNA was isolated from leaf tissues of greenhouse-grown plants using the procedure of Lodhi et al. [23] with a modified extraction buffer containing an increased EDTA concentration of 100 mmol/l. Genotypes used to obtain target promoter sequences included five *V. vinifera* genotypes ('Merlot', 'Pinot Noir', 'Pinotage', 'Remaily Seedless' and 'Superior Seedless'), a wild *Vitis* species 'Haines City' (*Vitis shuttleworthii* House) and a Florida bunch grape hybrid 'BN5-4′ [*Vitis aestivalis* Michx. ssp. *simpsoni* Munson X 'Remaily Seedless' (*V. vinifera* X *Vitis labrusca* L.)] (developed at the Florida Experiment Station, University of Florida).

Oligonucleotide primers were designed by using Vector NTI Advance™. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Gateway Clonase Technology (InVitrogen) was utilized to facilitate the cloning of PCR-amplified DNA fragments into transformation vectors. Accordingly, DNA sequences corresponding to both attB1 (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CT-3′) and attB2 (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT-3′) sites for BP recombination reaction were incorporated into the terminal region of forward and reverse primers respectively (Table 2).

Chemical reagents and DNA polymerase were purchased from Promega (Madison, WI, USA). PCR reactions were carried out in