FINAL REPORT

GRAPE & WINE RESEARCH & DEVELOPMENT CORPORATION

Project Title: Isolation of gene promoters required for controlling genes in grapevine

Project No: CSH 95/1

Organisation Commonwealth Scientific and Industrial Organisation (CSIRO)

Location: CSIRO Horticulture, Hartley Grove, Urrbrae, SA 5064

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Summary

Genetic improvement of grapevine varieties by molecular biology techniques requires the availability of appropriate genes, a system to introduce these genes into grapevines and the use of suitable gene promoters which are the molecular switches that control the level (expression) and site (tissue) of gene activity in plants. This project involved the isolation of gene promoters for targeted expression of genes to the grape berry. The berry-specific promoters isolated from grapevine represents an important part of an overall strategy to genetically manipulate and improve existing grapevine varieties. Specific genes expressed at different stages of berry development were (successfully identified and the putative genomic DNA promoter regions controlling these genes were successfully isolated. Research on these promoter regions is continuing as part of a new grapevine genetic improvement program with the development of plant expression cassettes for transformation into grapevine to enable testing of tissue-specific expression pattern sin *planta.* After testing the promoters will be available for use by parties involved in the new Australian collaborative grapevine genetic improvement program to genetically improve existing grapevine varieties.

Background

Genetic improvement of classic grapevine varieties by molecular biology depends on the simultaneous development of three key research areas:

(a) isolation of important grapevine genes
(b) methods for introducing these genes into plants and
(c) controlling the activity of induced genes with gene promoters.

The first two key areas are being actively researched by current CSIRO Division of Horticulture projects and CRC for Viticulture projects. The third key research area, controlling the activity and tissue specificity of introduced gene-by-gene promoters, had not been addressed and was the focus of this project. Without tissue-specific gene promoters the potential improvements through genetic manipulation of grapevine productivity, grape quality and disease resistance will not be fully realised.

Objectives

The project objective was to isolate and characterise suitable gene promoters from grapevine, which could be used to target gene expression to the grape berry.

Introductory technical information

At the time of project initiation there was no history of R&D in the literature in the area of gene promoter isolation and characterisation from grapevines, though this area was being actively pursued for other crops. Relevant research experience of the principal investigators specific to grapevine molecular biology was applied to this project as well as molecular technology and techniques from other sources and species.

Research methodology

Grapevine RNA was isolated from different tissue and the technology of differential display was applied to identify and isolate genes that showed putative berry-specific expression. The genes were characterised by northern analysis and DNA sequencing to determine berry specificity and putative function. Candidate genes that were berry-specific, including the polyphenol oxidase (PPO) gene were then used to recover promoter regions either from screening grapevine genomic libraries or by a PCR based promoter walking technique. The promoter regions were characterised by DNA sequence analysis. Development of a transient expression system for grapevine would allow rapid functional analysis and testing of the isolated promoter regions. A β-glucuronidase (GUS) reporter system was used in a transient transformation system using either *Agrobacterium* or biolistics as a delivery mechanism.

Results

The initial screening of grapevine tissues for cDNA clones, which demonstrate berry-specific expression patterns, identified two potential candidates:

- A MADS-box gene which is specifically expressed in floral tissues and in pre-veraison berries (See Figures 1 and 2).
- A Chitinase gene, which is specifically induced in berries at veraison and is highly expressed throughout berry ripening.

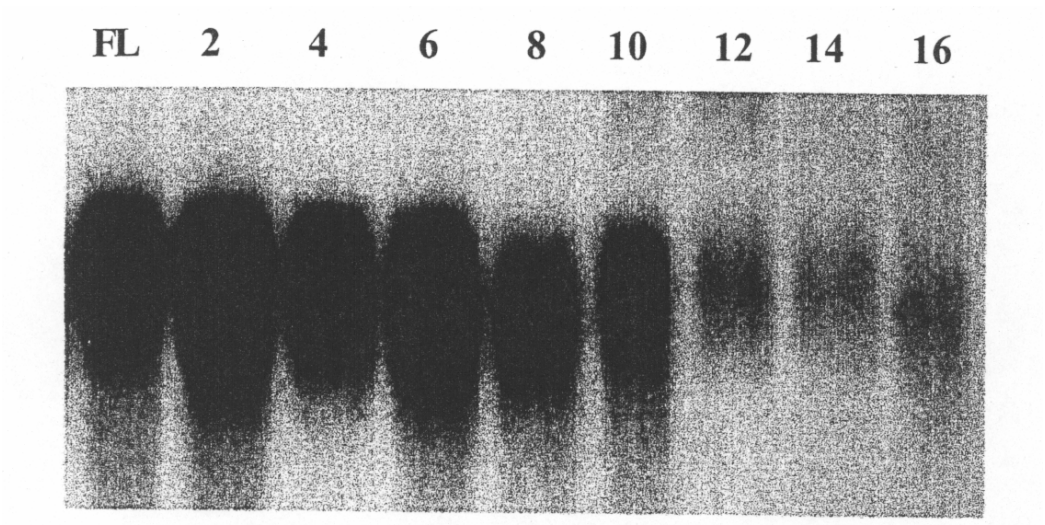

Figure 1: Northern analysis for gene expression of the grapevine MADS-box gene in Shiraz. FL (flowers). Berries 2 -16 weeks after anthesis. Veraison occurred between weeks 10 and 12. Gene expression is strong in flowers and pre-veraison berries and weak in post-veraison berries.

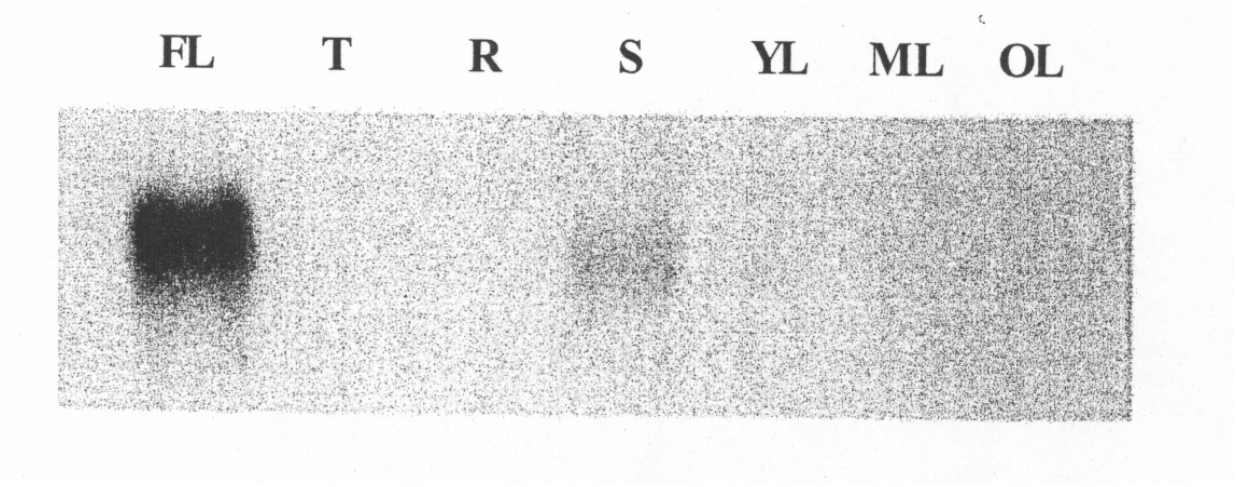

Figure 2: Northern analysis for gene expression of the grapevine MADS-box gene in Shiraz. FL (flowers) T (tendrils), R (roots), S (seeds), YL (young leaf), ML (Mature leaf), OL (old leaf). The gene is specifically expressed in reproductive tissue and not vegetative tissue.

For the isolation of DNA promoter regions controlling the expression of these genes, high molecular weight DNA was prepared from both Sultana and Shiraz tissue and two separate large insert genomic libraries were constructed, Screening of these libraries for genomic fragments resulted in the isolation of a number of closely related sequences but did not yield the specific clones of interest, A PCR-based method (promoter-walking) was then adopted and this resulted in the successful isolation of genomic fragments for both of the berry-specific promoters from both Sultana and Shiraz. The genomic DNA fragments, ranging in size from 1.4-1.8 kbp, have been fully sequenced and the putative promoter regions identified. Promoter fragments have also “been isolated for the polyphenol oxidase gene which causes browning reactions in wine and dried fruit. Other interesting genes expressed in the berry were identified during the isolation of putative (' berry specific genes and include a heat shock protein gene which appears to be both developmentally regulated in the berry and heat induced.

Appendix 2 contains commercial-in-confidence DNA sequence information of the promote fragments and genes.

An experimental system was initially investigated in an attempted to develop methods for the rapid testing of promoter constructs in grapevine leaves and berries by the use of transient assays employing the β glucuronidase (GUS) reporter gene under the control of the cauliflower mosaic virus 35S promoter. Both biolistic and *Agrobacterium-based* methods were tested for the introduction of GUS constructs into berries and leaves of grapevine and leaves of tobacco. While we were able to demonstrate successful transient GUS expression in tobacco leaves, grape leaves proved to be much more difficult and expression patterns" were not reproducible. Assays involving grape berries were further complicated by the presence of significant levels of endogenous GUS activity indicating that this reporter gene is not suitable for assays involving berry tissue. Alternative reporter genes, which may be better suited for use in grape tissues, are now in the process of being evaluated with the green fluorescent protein (GFP) reporter gene showing promising results.

Discussion

This project has successfully identified berry-specific genes, which are expressed at different stages of berry development and putative genomic DNA promoter regions controlling these genes have been isolated. Characterisation of promoter regions by a transient assay using the GUS reporter gene proved to be unsuccessful as a result of the chosen grapevine tissue not being amenable to the GUS assay. Characterisation of the promoter regions is continuing with the use of a new reporter gene, GFP. The research has now been incorporated into a larger program on grapevine genetic improvement which has the support of both industry (DFRDC, GWRDC) and research bodies (CSIRO, University Adelaide, CRC for Viticulture).

The project attracted international interest with the visit in 1996 by an overseas scientist, Dr. S. I. Matsumoto from Japan to work in the laboratory. While publications and presentations have I' been restricted by the nature of the work and potential intellectual property considerations the following are recorded:

Iocco P, Franks T, Jacobs A, Boss P, Davies C, Dry IB, Robinson SP, Scott NS, Thomas MR (1996) Towards genetic manipulation of the grape berry. The organisation and expression of the genome, 19th Annual Genome Conference, Lome, Australia,

Matsumoto S, Dry ,IB, Thomas MR., (1997) Nucleotide sequence of grapevine *(Vitis vinifera)* cDNA similar to SNAP proteins. DNA sequence 8: 109-112

Please see appendix 1 for text of publications.

Implications and recommendations
The aim of this project was to isolate and characterise berry-specific promoter fragments for use in future projects that aim to improve grapevine varieties through gene modification. The results of this research has now been incorporated into a larger genetic improvement program known as "Grapevines for the new Millennium" and research on tissue-specific promoter isolation and use will continue in this program. "

Intellectual Property
Funding Contributions of each Partner

Partners	1994/95	1995/96	1996/97	TOTAL ($)
GWRDC	-	24,670	28,509	53,179
DFRDC	31,144	24670	28,509	84,323
CSIRO	180,734	180,374	135,810	497,278
TOTAL ($)	211,878	230,074	192,828	634,780

Based on the above table the Corporation and the Research Organisation agree that the Corporation's share of title to all intellectual property will be 8.4%.

A decision will be made, with industry consultation, on how best to protect and use the tissue-specific promoters for the benefit of the Australian industry once the promoter regions have been fully tested and characterised by either transient or stable transformation experiments. This will likely occur within the framework of the "Grapevines for the new Millennium" program where the research is continuing. The value of the technology is the ability to control the tissue-specific expression of introduced genes in transgenic grapevines. At this point in time it is difficult to reliably quantify the financial value.

Appendix 2 contains commercial-in-confidence DNA sequence information of the promoter fragments.

Technical Summary
New molecular methods, skills and resources, such as differential display technologies, grapevine genomic libraries and promoter-walking libraries were developed during the period of this project.

These will be utilised in future molecular projects aimed at improving grapevine varieties through gene modification.

Appendix 1 Publications (only one included in this document)

Appendix 1

TOWARDS GENETIC MANIPULATION OF THE GRAPE BERRY

Pat Iocco, Tricia Franks, Andrew Jacobs, Paul Boss, Chris Davis, Ian Dry, Simon P. Robinson,

Nigel S. Scott **and Mark R. Thomas**

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Grapevine is a vegetatively propagated crop with the industry based on only a few traditional cultivars. Genetic manipulation of these traditional cultivars is of particular relevance as a means of genetic improvement due to the low acceptance of new varieties produced by conventional breeding.

We are identifying and understanding the role of genes specifically expressed during berry development, identifying and isolating promoters that control the temporal and spatial expression of these genes and developing a transformation system to introduce genes.

Berry development is divided into two main stages. A pre-veraison stage, when the berry is green and D hard and a post-verasion stage where the berry softens and colours. Genes have been isolated and characterised that show developmental expression contained to either the pre-verasion stage or the post-verasion stage, including polyphenol oxidase, invertase, chitinase, a heat shock gene and a U MADS-box gene. Characterisation of relevant promoter regions is proceeding with the goal of producing expression vectors for targeting synthetic gene expression and gene silencing to the grape berry.

Genetic manipulation relies on producing transgenic grapevine plants of commercial cultivars. We have recently developed a system for transforming grapevine using *Agrobacterium tumefaciens.* The system is based on the use of embryogenic cultures, which regenerate plants with the expected DNA profile but exhibit a phenotype characteristic of juvenile plants. The usual time frame from a juvenile to mature stage is three years and corresponds to the appearance of fruit. This long time period until -flowering has obvious implications for the project and represents a current bottleneck in assessing modified berry specific genes.

Abstract for The Organisation and Expression of the Genome. 19th Annual Genome Conference. Lorne, Australia, 1996.