

# Grape (*Vitis vinifera* L.) BAC Library Construction, Preliminary STC Analysis, and Identification of Clones Associated with Flavonoid and Stilbene Biosynthesis

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We have constructed a grape BAC library using the cultivar Syrah. The library contains 55,296 clones stored in 144 384-well microtiter plates. A random sampling of 381 BACs indicated an average insert size of 144 kb with a range of 30 to 355 kb, and less than 4% of the clones do not contain inserts. Eighty-nine percent of BAC clones in the library have an average insert size greater than 100 kb. Based on a genome size of 483 Mb, library coverage is 16.5 haploid genome equivalents. Screening the BAC library colony filters with cpDNA sequences showed that contamination of the genomic library with chloroplast clones was low (1.5%). Library screening of an 11X coverage (2 BAC colony filters) with 12 cDNA probes corresponding to flavonoid and stilbene biosynthesis genes resulted in an average of 13 hits per probe (range = 1 to 27). To gain a glimpse into the grape genome and evaluate the library for sequence-tagged connector (STC) development, 768 BAC clones were end sequenced in both forward and reverse directions. The STCs were queried against the SWISS-PROT database and significant hits were sorted according to putative function.

**Key words:** Grape, BAC library, BAC end sequencing, flavonoid and stilbene biosynthesis

Grape (*Vitis vinifera* L.) is one of the most important horticultural fruit crops in the world [13]. Rapid progress in cultivar improvement through traditional breeding methods is hampered by extensive labor, funding, and other aspects required for cultivation. Furthermore, our understanding of grapevine genetics is limited, primarily due to the lack of inbred lines required for extensive genetic study. Therefore, molecular breeding approaches provide additional tools in the development and subsequent selection of novel grape cultivars.

In recent years, the molecular mapping of the grape genome has been accomplished using dominant PCR-based markers and a pseudotestcross approach to linkage analysis [4,7]. These maps are able to provide the necessary guide for the physical mapping and cloning of genes associated with important horticultural traits.

The map-based cloning of important genes requires the development of large insert genomic libraries. The bacterial artificial chromosome (BAC) cloning system appears to offer advantages over other large insert cloning systems [11,18]. Therefore,

the BAC system is very appealing as a vehicle for advanced genome analysis in grape. At the present time, there are no published reports of BAC libraries for grape.

In the current study, we report the development of a BAC library for the grape cultivar Syrah, an important red wine germplasm that contains genes of interest in regard to flavonoid and stilbene biosynthesis. The library was characterized with chloroplast DNA sequences and cDNAs associated with flavonoid and stilbene biosynthesis. A preliminary STC database for the grape genome based on the sequencing of 1,536 BAC ends was also developed. Bioinformatic analysis of the STCs provides new insights into grape genome structure.

## Materials and Methods

**BAC library construction.** A new BAC vector, pCUGI-1 was used and prepared as described by Luo et al. [8]. Megabase plant DNA embedded in agarose plugs was obtained as described by Peterson et al. [9], using the method for plant tissue containing high levels of secondary compounds. Partial digests of megabase DNA (using *HindIII*, size selections, and ligations were performed as described in detail by Peterson et al. [9]. Re-combinant colonies were picked using the Genetix Q-bot (Genetix Corp., Queensway, UK) and stored individually in 144 384-well microtiter plates. Three copies of the library were made and stored in separate -80°C freezers.

**BAC clone characterization.** To prepare BAC DNA, 3 mL LB chloramphenicol (12.5 ug /uL) cultures were grown overnight in six-cell autogen tubes and miniprep robotically (Autogen 740 plasmid isolation system, Framingham, MA). To

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estimate insert size and determine distribution of clone size, a total of 384 BAC preps were performed from clones selected at random throughout the library. Due to DNA sample loss, 381 preps were used for restriction analyses. The BAC DNA was digested with 7.5 units (10 hr at 37°C) of *NotI* and analyzed by pulsed field electrophoresis in 1% agarose gels (6 v/cm, 5 to 15 sec switch time, 15 hr run time, 14°C). Southern blots of size-separated BAC inserts were performed using standard protocols [10] after UV nicking the gels (Gene Linker, Bio-Rad, Hercules, CA). Total genomic grape DNA for use as probe was extracted from Syrah plants using the DNAzol® ES extraction protocol for plants (Molecular Research Center, Cincinnati, OH) and <sup>32</sup>P labeled using standard random priming techniques [10].

**BAC library screening.** High-density colony filters for hybridization based screening of the library were prepared using the Genetix Q-bot. Clones were gridded in double spots using a 4x4 array on 22.5 cm square Hybond N+ filters (Amersham Corp., Buckinghamshire, UK). This gridding pattern allows 18,432 clones to be represented per filter. Colony filters were treated and hybridized using standard techniques [10]. Radiolabeling (<sup>32</sup>P) of probe DNA and hybridization of colony filters was performed using standard techniques [10]. Screening for chloroplast DNA in the library used three barley chloroplast clones containing *ndhA* (470 bp), *rbcl* (1,300 bp), and *psbA* (1,400 bp) sequences. These sequences are spaced equidistant around the 133 kb barley chloroplast genome. Chloroplast clones were obtained from J. DuBell (Dept. Biochemistry and Biophysics, Texas A&M University, College Station). Screening was also performed with 12 cDNA clones associated with flavonoid and stilbene biosynthesis as indicated in Table 1. A summary of this pathway and the various genes involved is presented by Sparvoli et al. [12]. The cDNA clones used for the BAC filter hybridizations were obtained from a grape EST project managed by the DuPont Corporation (Wilmington, DE). The putative function of each cDNA was determined based on sequence similarity.

**BAC end sequencing.** Preparation of BAC DNA for end sequencing was done in a 96-well format using standard alka-

line lysis miniprep techniques. Sequencing reactions were set up according to the manufacturer instructions for the Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA). Reactions were performed using forward and reverse universal primers. Samples were loaded onto 48-lane sequencing gels in ABI377 automated sequencers. Gels (250 mL) were composed of the following: 5% Long Ranger (FMC), 6M urea, TEMED 18 uL, 150 uL ammonium persulfate (10% stock), and 1x TBE buffer. Reaction products were electrophoresed using a 3.5 hr run. Base-calling was performed automatically using PHRED [5,6], and vector sequences were removed by CROSS-MATCH (<http://www.genome.washington.edu>). High-quality BAC end sequences (defined as those having >100 nonvector bases with a PHRED quality value >20) were used as queries in FASTX searches of the SWISS-PROT database [2]. All software was run locally on a Sun Ultra30 workstation using Solaris 2.6. The grape BAC end sequences have been submitted to GenBank under accession numbers BH1676222746551 to BH1686692747586.

## Results

**BAC library construction and characterization.** We have constructed a BAC library for the red winegrape cultivar Syrah that is suitable for physical mapping, DNA sequencing, and cloning genes associated with key horticultural traits. *HindIII* was used as the cloning enzyme. The library consists of 55,296 clones stored in 144 384-well microtiter plates. Less than 4% of the clones do not contain inserts as judged by random analysis of BACs sampled from the library. A random sampling of 381 BACs taken from the library indicated an average insert size of 144 kb, with a range of 30 to 355 kb. Based on a haploid genome size of 483 mb [1], the coverage of the library is approximately 16.5 haploid genome equivalents, resulting in a 99% probability of recovering any specific sequence. Figure 1 shows 23 randomly selected clones digested with *NotI* to release the insert. The two *NotI* sites in pCUGI-1 flank the multicloning site. Because *NotI* is a GC-8-base cutter and the grape genome is relatively AT rich, digestion typically generates a vector band plus one insert band.

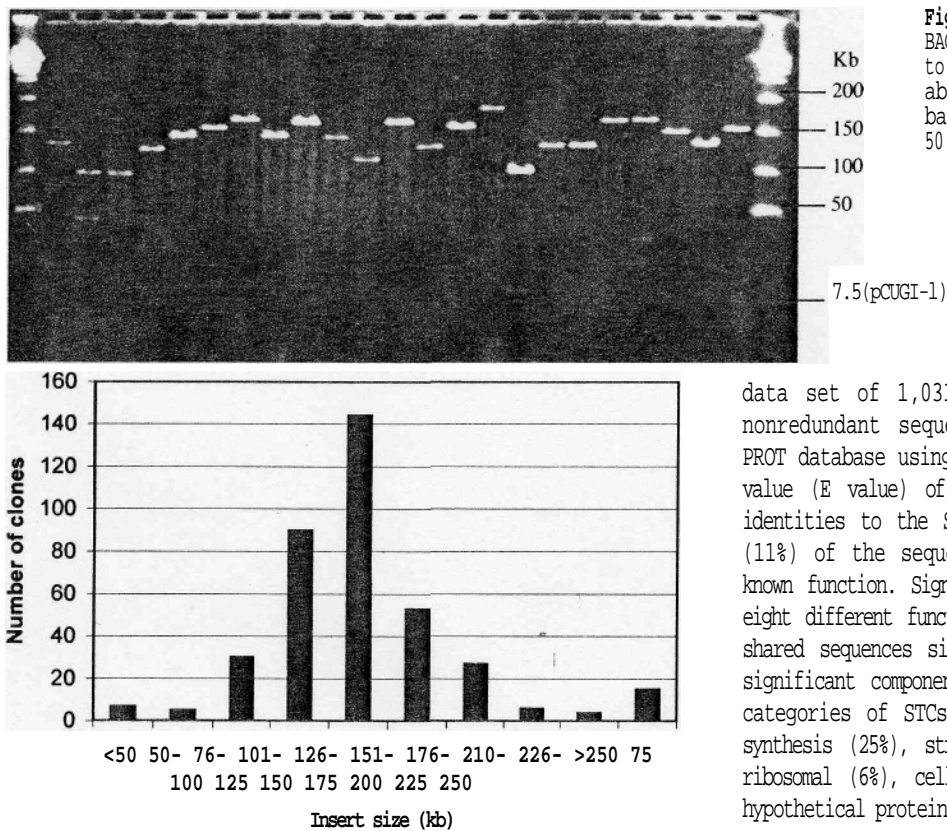
To determine the size distribution of BAC clones in the library, the 381 BACs analyzed with *NotI* digests were grouped by insert size, and the insert size of each clone was plotted against the frequency of each group of clones represented in the library (Figure 2). Based on this analysis, 89% of the clones in the library have an average insert size greater than 100 kb. Of the clones larger than 100 kb, 65% are greater than 125 kb.

To obtain an estimate of the representation of chloroplast DNA in the library, colony filters were screened with three different chloroplast genes. Results from this screening showed that approximately 1.5% of library sequences are chloroplast DNA (data not shown). The low chloroplast DNA content of the library is likely due to the use of nuclei as a megabase DNA source rather than protoplasts [14,15,19].

To test the library for coverage and isolate genomic regions associated with stilbene and flavonoid biosynthesis, screening of high-density BAC colony filters was performed using 12 grape cDNAs identified as representing genes associated with the stil-

**Table 1** Grape BAC library hybridization results using 12 cDNA probes associated with anthocyanin biosynthesis. Two high-density BAC colony filter arrays were used for each probing, allowing the screening of 11 haploid genome equivalents.

Probe	No. hits
CHS (chalcone synthase)	27
StSy (Stilbene synthase)	15
DFR (dihydroflavonol 4-reductase)	6
CHR (Chalcone reductase)	22
F3H (flavanone 3-hydroxylase)	24
ANS (Anthocyanin synthase)	6
CHI (chalcone isomerase)	9
F3'H (Flavonoid 3',5'-Hydroxylase )	7
PAL (Phenylalanine ammonia lyase)	19
FLS (Flavonol synthase)	1
C4H (Cinnamate 4-hydroxylase)	21
F3'H (Flavonoid 3'5'-Hydroxylase (3'))	3



**Figure 1** Analysis of 23 randomly selected grape BAC clones. Ethidium bromide stained CHEF gel (5 to 15 sec switch time, 14 hr) showing insert DNA above the common 7.5 kb pCUGI-1 vector band. Molecular weight marker in outside lanes is a 50 kb lambda concatamer.

**Figure 2** Insert size distribution of BAC clones in the grape Syrah BAC library. To estimate insert size range, BAC DNA from 381 randomly selected clones were analyzed, as shown in Figure 1. Results indicate that the average insert size is 144 kb, with over 89% of the clones > 100 kb.

bene and flavonoid biosynthesis pathway (Table 1). The filter sets used for the cDNA-probes consisted of two filters representing 36,864 clones or 11 haploid genome equivalents. An average of 13 positive signals were obtained for the 12 probes, with a range of 1 to 27. The wide range of positive signals identified between probes is likely indicative of the effects of preferential cloning obtained from the use of a restriction enzyme to generate the inserts. Nevertheless, an adequate number of positive clones were obtained for all probes, except for one cDNA that only produced one positive signal. The average number of hits was slightly above the expected estimate of 11 positive signals per probe, suggesting that some of the cDNAs may have targeted duplicated regions of the genome or members of gene families. The BAC clones identified from this effort will provide the full gene sequence including regulatory regions for many of the key proteins involved in flavonoid and stilbene biosynthesis.

**BAC end sequencing.** To examine the feasibility of using a STC strategy [17] to establish a framework for sequencing selected regions of the grape genome, we sequenced and analyzed the ends (forward and reverse) of the first 768 clones in the library. After editing, redundancy was evaluated by querying the sequences against themselves, with the result that a nonredundant

data set of 1,031 sequences was developed. High-quality nonredundant sequences were searched against the SWISS-PROT database using the FASTX algorithm. A probability cutoff value (E value) of at least  $10^{-6}$  was used to assign putative identities to the STCs. The SWISS-PROT search resulted in 110 (11%) of the sequences showing similarity to sequences of known function. Significant search results were then sorted into eight different functional categories (Table 2). Many of the STCs shared sequences similar to retrotransposons and constituted a significant component of the data set (41%). The next largest categories of STCs were those involved in metabolism/photosynthesis (25%), structural roles (14%), regulatory roles (9%), ribosomal (6%), cell defense, communication, division (3%), and hypothetical proteins (1%).

The highly significant STCs showing similarity to various plant proteins other than retroelements were extracted from the data set and are listed in Table 3. Of these STCs, 18% were best matches to *Arabidopsis thaliana* proteins, likely because both are dicots and *Arabidopsis* is the most-studied plant species whose genome has been sequenced. The hits on *Arabidopsis* proteins contained a variety of functions in the metabolic, regulatory, structural and ribosomal categories. Other plant species giving multiple best matches included a variety of dicots and monocots: *Nicotiana tabacum* (10%), *Zea Mays* (10%), *Glycine max* (8%), *Lycopersicon esculentum* (8%), *Oryza sativa* (8%), and *Solanum tuberosum* (8%). The other putative plant protein functions were based on matches from 11 other diverse plant species.

**Table 2** Results from query of grape BAC end sequences against the SWISS-PROT database. Significant hits ( $N = 110$ ,  $E < 1 \times 10^{-6}$ ) were categorized according to function and listed in descending order with largest group first.

Functional category	No.	Percent
Retroelement related	45	41
Enzymatic(metabolic,photosynthesis)	28	25
Structural	16	14
Regulatory(kinase,phosphatase,transcriptional)	10	9
Ribosomal	7	6
Cell defense, communication, and division	3	3
Hypothetical/unclassified	1	1

## Discussion

Here we describe the development and characterization of a high-quality BAC library for the red winegrape cultivar Syrah. This large insert library provides an important resource for map-based cloning of genes, physical mapping, and DNA sequencing in this valuable fruit crop. The library has been deposited in the Clemson University Genomics Institute BAC/EST Resource Center and is publicly available upon signing a material transfer agreement with the Centre for Plant Conservation Genetics. Requests for high-density BAC colony filter arrays and clones can be submitted through the Clemson University Genomics Institute web page ([www.genome.clemson.edu](http://www.genome.clemson.edu)).

The utility of the library for identifying important regions of the grape genome associated with color development in the berry

was demonstrated by screening the BAC colony filters with 12 cDNAs representing key enzymes in the flavonoid and stilbene biosynthesis pathway. The flavonoid biosynthetic genes were chosen because they control important traits such as disease resistance, fruit color, and production of secondary metabolites, which provide benefits to human health. Most of these genes have been shown to be present in single or low copy number in many plant species, including grape [12]. Therefore, the presence and frequency of these genes within the grape BAC library provides some indication as to the coverage of the library. CHS genes are generally present in higher copy number than other flavonoid genes, and it has been shown that StSy genes are present in greater numbers [12]. Probes used to screen the BAC library were from expressed gene sequences and may have cross-hybridized to multiple gene family members of high sequence

**Table 3** The highly significant hits ( $E < 1 \times 10^{-6}$ ) for plant proteins (excluding retroelement and proline-rich proteins) from the SWISS-PROT query of grape BAC end sequences. Entries are sorted alphabetically by best match plant species and secondly by significance. End sequences have been submitted to GenBank under accession numbers BH1676222746551 to BH1686692747586.

BAC end ID	Best match protein	Best match plant species	% identity	Significance
1J07f	ATP synthase alpha chain	<i>Arabidopsis thaliana</i>	92	8.5e-73
2P08r	60S ribosomal protein L21	<i>Arabidopsis thaliana</i>	86	4.2e-48
2G15f	Cytochrome P450	<i>Arabidopsis thaliana</i>	61	6.7e-46
2N20r	Calnexin homolog precursor	<i>Arabidopsis thaliana</i>	59	7.4e-34
1I02f	Glycine-rich cell wall protein	<i>Arabidopsis thaliana</i>	53	9.8-11
2M17f	Histidine transporter protein	<i>Arabidopsis thaliana</i>	50	2.9e-09
2N22r	Protein kinase TMK1	<i>Arabidopsis thaliana</i>	33	1.9e-06
1L21r	Cationic peroxidase	<i>Arachis hypogaea</i>	34	1.9e-06
1k15f	Fructose-1,6- bisphosphate	<i>Brassica napus</i>	75	2.3e-20
2D04r	Cytochrome P450	<i>Catharanthus roseus</i>	40	1.1e-07
2E15r	Dihydroflavonol-4-reductase	<i>Gerbera hybrida</i>	39	2.3e-10
2N16f	Potential heme binding protein	<i>Glycine max</i>	73	5.4e-48
1D17f	Nodulin 21	<i>Glycine max</i>	64	6.7e-23
2D24F	Early nodulin 75 precursor	<i>Glycine max</i>	34	2.9e-11
1117r	Delta cadenine synthase isozyme XC14	<i>Gossypium arboreum</i>	52	1e-19
2n12r	23KDA Jasmonate induced protein	<i>Hordeum vulgare</i>	91	2.6e-58
2N10r	Heat shock 70KDA protein	<i>Lycopersicon esculentum</i>	64	2e-36
1B07r	Water stress induced tonoplast protein	<i>Lycopersicon esculentum</i>	66	1.9e-13
2J09f	Plasma membrane ATPase proton pump	<i>Lycopersicon esculentum</i>	85	2.7e-08
2L02r	60S ribosomal protein L2	<i>Nicotiana tabacum</i>	80	5.4e-24
2C22r	Magnesium chelatase subunit	<i>Nicotiana tabacum</i>	83	2.2e-18
1L06r	NADH-plastoquinone oxidoreductase	<i>Nicotiana tabacum</i>	78	3e-13
1A08r	Aristolochene synthase	<i>Nicotiana tabacum</i>	31	4e11
2E12f	Fructose bisphosphate aldolase	<i>Oryza sativa</i>	66	8.9e-42
2F08r	40S ribosomal protein S19	<i>Oryza sativa</i>	86	6.9e-40
2F24r	60S ribosomal protein L5	<i>Oryza sativa</i>	69	2.4e-21
2M10r	Glycine-rich cell wall protein	<i>Petunia hybrida</i>	55	3.7e-24
1N15f	Desaturase	<i>Ricinus communis</i>	86	2.8e-15
2J17r	Phospholipase	<i>Ricinus communis</i>	59	9e-06
2B17r	Photosystem II Chlorophyll A	<i>Populus deltoides</i>	50	4.3e-25
2H02r	60S ribosomal subunit L5	<i>Solanum melongena</i>	67	1.8e-25
2A23r	Cytochrome P450	<i>Solanum melongena</i>	44	4.8e-13
2N24r	Photosystem II 10KDA	<i>Solanum tuberosum</i>	54	2e-10
2P02r	Photopsystem II 10KDA	<i>Spinacia oleracea</i>	63	3.7e-21
1D13r	Chloroplast 30S ribosomal protein 30S	<i>Spinacia oleracea</i>	67	1.5e-09
2P24r	2,3-bisphosphoglycerate	<i>Zea mays</i>	88	8.6e-57
2H16r	60S Acidic ribosomal protein	<i>Zea mays</i>	83	6.9e-28
2L24r	Histone H2A	<i>Zea mays</i>	68	1.1e14
2B03r	Tubulin beta-1 chain	<i>Zea mays</i>	53	6.6e-20

similarity. The results, however, were indicative of the good representation provided by the library, as an average of 13 hits per probing were obtained when an 11X filter representation was screened.

The BAC library screening results also suggest a close physical linkage of the StSy and CHS genes in the grape genome: 19 BAC clones hybridized to CHS only, 7 hybridized to StSy only, and 8 hybridized to both CHS and StSy. Tropf et al. [16] have suggested that StSy genes have developed from CHS genes several times in the course of evolution. The close physical association of CHS and StSy genes in grape may be the result of a CHS gene duplication event, giving rise to a StSy gene.

End sequencing of 1,536 BAC clones produced a nonredundant high-quality set of 1,031 STCs. Search results against the SWISS-PROT database gave 110 significant hits ( $> 1 \times 10^{-6}$ ) that were sorted according to function. Not surprisingly, many of the matches were to retroelement related sequences (41%). This is actually a fairly low level of retroelement content for a dicot. In a preliminary survey of BAC end sequences for tomato, a higher level (48%) of retroelement matches were found [3]. Because of the small genome size for grape and the low level of retroelement content, the grape BAC library is ideally suited for the development of an STC framework.

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