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Two new grape cultivars, bud sports of Cabernet Sauvignon bearing pale-coloured berries, are the result of deletion of two regulatory genes of the berry colour locus

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Abstract Bud sports are infrequent changes in phenotype affecting shoots of woody perennials but the molecular basis of these mutations has rarely been identified. In this report, we show that the bronze-coloured berries of the Malian cultivar, a documented bud sport of the wine grape Cabernet Sauvignon (*Vitis vinifera* L.), lack anthocyanins in the subepidermal cells compared to the red/black berried Cabernet Sauvignon in which both the epidermis and several subepidermal cell layers contain anthocyanin. The Malian phenotype is correlated with an alteration in the genome indicated by a reduction of hybridisation signal using a *MYBA* probe. In Shalistin, a white-berried bud sport of Malian, the red allele at the berry colour locus appears to have been deleted completely. These data suggest that Malian could be a L1/L2 periclinal chimera, which gave rise to Shalistin by an invasion of epidermal cells (L1) by the mutated subepidermal cells (L2). The red grape Pinot Noir has given rise to a number of pale coloured sports, although the provenance of the extant sports is not known. We show that a clone of Pinot Blanc (white-berried) does not have a deletion of the red allele of the same dimensions as that in Shalistin, though a small deletion is a likely explanation for the altered phenotype. However, the mechanism of deletion of the red allele of the berry colour locus is a possible means by which other red to white clonal mutations of grapevines have occurred.

Keywords Anthocyanin · Bud sports · Genetic instability · Periclinal chimera · Wine grapes

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Introduction

Some woody perennial plants such as apples and grapes have a high degree of heterozygosity in their genome (Kenis and Keulemans 2005; Olmo 1976; Aradhya et al. 2003); therefore, it is very difficult to recreate a cultivar by sexual propagation. Propagation is achieved by vegetative techniques such as by cutting, grafting and budding, because the genetic characteristics of the cultivar are maintained resulting in many plants of a very similar phenotype. However, on rare occasions, some cultivars can demonstrate unstable phenotypes resulting in a portion of the plant, sometimes extending to whole branches, having different characteristics. When these branches (bud sports) are vegetatively propagated by clonal techniques, the new phenotype is generally maintained leading to a new variety, often exhibiting only one phenotypic character different from the parent (Marcotrigiano 1997). Horticulturists propagate bud sports because they continue to exhibit all of the other desirable characteristics of the parent (Franks et al. 2002).

Among the bud sports, colour mutants with alterations in the red or purple anthocyanin content of flowers or fruit are common and examples in horticultural plants are widespread. For example, there have been at least 13 flower colour mutants of the yellow-pink rose, Peace

(Zykov 2000) of which the deeply coloured Chicago Peace and Flaming Peace are the best known sports. Two apple cultivars, Royal Gala and Galaxy, are bud sports of Gala with more highly coloured fruit than the original parent (Dickinson and White 1986). Alterations in the anthocyanins of a plant can be very distinct and easily observed and generally have no detrimental effect on the viability of the plant in the garden or orchard.

The molecular basis behind the generation of sports in woody perennial plants is not well understood. Transposable elements, first recognised by Barbara McClintock in maize (McClintock 1951), have been identified in many species including petunia (van Houwelingen et al. 1998) and morning glory (*Ipomoea* sp.) (Clegg and Durbin 2000) in which the mobility of the elements can be responsible for changes in flower colour.

Anthocyanins are red and purple secondary metabolites, synthesised by the flavonoid biosynthetic pathway. This pathway branches to form the colourless flavonols and condensed tannins, which can oxidise to a brown colour. The pathway has been studied extensively in many plant species at the molecular level and genes encoding the enzymes of the pathway have been cloned and characterised. Activity of the pathway is controlled by a transcription complex composed of three regulatory proteins belonging to the MYB, HLH and WD40 classes (Koes et al. 2005), which activate the structural genes in the flavonoid pathway. Specific Myb proteins regulate the synthesis of each of the different flavonoid compounds whereas the HLH and WD40 proteins also regulate other processes in the plant such as vacuolar pH or hair initiation. Grapes have two *MYB* genes which regulate colour (Kobayashi et al. 2002), one of which is inactivated by a retrotransposon in white grapes (Kobayashi et al. 2004; 2005).

The ancestral plants of *Vitis vinifera* L. (grapevine) are thought to have produced red/black coloured berries (Slinkard and Singleton 1984). Most white grape cultivars arose from a single red-berried parent by the accumulation of different mutations in two adjacent *MYB* genes encoding similar transcription factors, resulting in a white skin allele, followed by sexual propagation (Walker et al., submitted). Since both *MYBs* are functional, both must be inactivated to produce white grapes.

On rare occasions bud sports bearing different coloured berries have been noted in a number of grape cultivars, reds going through bronze, rose or grey to white although the provenance of the plants is not usually known. For example, it is likely that Pinot Noir has given rise to both Pinot Gris or Pinot Grigio (grey) and Pinot Blanc (white) by mutation of unidentified gene/s (Hocquigny et al. 2004); however, the actual parental vines, on which the sports arose, are no longer available. They also conclude that Pinot Meunier arose from Pinot Gris, while a more likely explanation is that the Pinot Noir parent of the closely related Pinot Gris and Pinot Meunier vines is not represented in their collection, resulting in several hypotheses for the evolution of the Pinot cépage (family of cultivars).

Dark berried Cabernet Sauvignon is heterozygous for colour, carrying one red and one white allele of the berry colour locus (Walker et al., submitted), and is probably the result of a cross between Cabernet Franc (red) and Sauvignon Blanc (white) (Bowers and Meredith 1997). In a vineyard planted with *Cabernet Sauvignon* at Langhorne Creek, South Australia in 1977, a vine bearing both red/black bunches and bronze grape bunches was observed. Close examination revealed a single cane with mutant bronze coloured berries, suggesting that the change in berry colour was the result of a bud sport. When the mutant cane was propagated vegetatively, the mutant bronze berry phenotype was maintained through several rounds of vegetative propagation and the mutant was registered as the new cultivar, Malian (Cleggett 2002). In 1991 in a small planting of Malian, a cane bearing white berries was noticed and propagated vegetatively by cane cuttings to produce the cultivar Shalistin described in Cleggett (2003). Boss et al. (1996) showed that Malian or Bronze Cabernet berries, had 90% less anthocyanin than Cabernet Sauvignon fruit while Shalistin (White Cabernet) berries contained no anthocyanin and had a corresponding reduction in the expression of the many of the genes of the flavonoid pathway.

In this report we investigate the molecular basis for the change in colour in the Cabernet bud sports and Pinot colour sports, finding that deletion of the berry colour locus in different cell layers is the most likely cause of the phenotypes and propose a model for how these alterations can give rise to the colour sports of Cabernet Sauvignon. Although the nature of the mutation at the colour locus differs between our clones of Pinot Gris and Pinot Blanc, the mechanism of deletion could be responsible for other bud sport phenotypes.

Materials and methods

Plant material

Young leaves and ripening berries of Cabernet Sauvignon, Malian and Shalistin were collected from grapevines grown in a commercial vineyard at Langhorne

Creek, South Australia, which also provided material for photography. Leaves for DNA extraction were frozen in liquid nitrogen and stored at –80°C.

Young leaves from other grape cultivars were collected from Coombe Vineyard, Urrbrae, South Australia and Nuriootpa Agricultural Centre, South Australian Research and Development Institute, snap frozen in liquid nitrogen and stored at –80°C. Red-fruited cultivars: Pinot Noir, Chambourcin, Durif, Malbec. Bronze-fruited cultivar: Pinot Gris. White-fruited cultivars: Pinot Blanc, Biancone, Calmeria, Canada Muscat, Canocazo, Chardonnay, Chasselas, Chenin Blanc, Clairette Blanche, Colombard, Crouchen, Dawn Seedless, Doradillo, Farana, Himrod, Italia, Marsanne, Merbein Seedless, Morio Muscat, Müller Thurgau, Muscadelle, Muscat Gordo Bianco, Ondenc, Orange Muscat, Palomino, Pedro Ximenez, Perlette, Riesling, Rkatziteli, Semillon, Seyval, Sugraone, Sultana, Taminga, Temprano, Traminer, Trebbiano, Verdelho, Villard Blanc, Viognier, Whaltham Cross, White Frontignac, White Muscat.

DNA methods

Using the method of Thomas and Scott (1993), genomic DNA was extracted from 1 g of very young leaves (between 1 and 3 cm in length), which had been ground to a powder in liquid nitrogen.

Genomic DNA (2 μg) was restricted overnight with 20 units of the appropriate enzyme at 37°C. Digests were electrophoresed overnight on 0.7% agarose gels in 1 × TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer. Southern blots were performed using the salt transfer method to Genescreen Plus® (Perkin Elmer Life Sciences) and hybridised overnight at 65°C with DNA probes labelled using Rediprime™ (Amersham Biosciences) with ^{32}P-dATP. The filters were washed in 2 × SSC + 1% SDS at 65°C for 10 min and then 0.1 × SSC + 1% SDS at 60°C for 10 min. Filters were exposed to Biomax MS film (Kodak) using Biomax (Kodak) intensifying screens at –80°C for 5 h to 2 days.

A Cabernet Sauvignon bacterial artificial chromosome (BAC) library (Donald et al., unpublished) was screened using PCR with the VvMybA primers (Kobayashi et al. 2002) and in subsequent rounds using identified BAC ends to build a contig of the berry colour locus (Walker et al., submitted) utilizing techniques described in Barker et al. (2005). BAC DNA was purified from a 100 ml overnight culture using a Qiagen midi-prep kit with the manufacturer's suggested modification of heating the elution buffer to 70°C to aid recovery of large DNA molecules.

PCR was conducted using 20 ng of genomic DNA plus Platinum Taq (Invitrogen) in accordance with the manufacturer's instructions in 50 μl reactions using cycling conditions of 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, followed by 72°C for 10 min. Where BAC templates (10 ng) were used, PCR was performed using BIOTA@ Red DNA polymerase (Bioline). The marker 20D18CB9 was scored using primers 20D18CB9f and 20D18CB9r (see Table 1) in a PCR, the product of which was restricted with *Dde*I and separated by gel electrophoresis. DNA for the probe for *MYBA* and for sequencing of *MYBA1* and *MYBA2* was amplified using primers MybAfw and MybArev. These primers amplify both red and white versions of these genes and *MYBA1* and *MYBA2* can be separated by gel electrophoresis based on size. Retrotransposon detection in the white allele was performed using PCR with primers VvmybA1a-c. DNA for the probe 53P11CB10 was obtained by PCR of BAC DNA using the primers 53P11CB10f and 53P11CB10r. The primer sequences are listed in Table 1.

Pulsed field gel electrophoresis (PFGE) was utilised to estimate the size of the BAC inserts. Using a Biorad PFGE apparatus, agarose gels (1% PFGE grade agarose, Biorad) was run for 18 h in 0.5 × TBE with a switch time of 1 up to 40 s at 6 V cm^{-1}, incline angle of 120° at 11°C.

For purification of DNA for sequencing and radiolabelling, DNA fragments were separated on 1 × TAE 1% agarose gels and extracted using Qiaex II gel extraction kit from Qiagen.

Sequencing was performed using BigDye version 3 (Applied Biosystems) in accordance with the manufacturer's instructions. Sequencing reactions were analysed at the Institute of Medical and Veterinary Science, Adelaide. Analysis of the sequence data was performed using GCG (Wisconsin) on WebAngis (Sydney).

Table 1 Sequence of primers

Name	Sequence
MybAfw[a]	cgctctagactcgatggagagcttaggagttag
MybArev[a]	cgcgagctctaaatcagatcaaatgatttactt
53P11CB10f	ttagtgactcgcacctccag
53P11CB10r	agaaacatagttttagtagggg
20D18CB9f	gatgaccaaactgccactga
20D18CB9r	atgaccttgtcccaccaa
MybA1fw5	tgcaacacagaaaaagggaacac
VvmybA1a[b]	aaaaaggggggcaatgtagggaccc
VvmybA1b[b]	ggacgttaaaaaatggttgcacgtg
VvmybA1c[b]	gaacctccttttgaagtggtgact

[a]After Kobayashi et al. (2002)

[b]After Kobayashi et al. (2004)

Microscopy

Oblique hand sections of ripening berries (between veraison and 3 weeks before harvest) were cut with a razor blade and floated on a water droplet. Samples were examined under a Zeiss Stemi 2000C dissecting scope and a Zeiss Axioplan light microscope and photos captured with a SPOT camera (Diagnostic Instruments Ltd.).

Results

Phenotype of Malian and Shalistin berries

The phenotype of the berries of the buds sports, Malian and Shalistin, which had originated from Cabernet Sauvignon, was examined. While Cabernet Sauvignon berries are very dark red, almost black in colour, Malian berries have a distinct bronze or pale pink colour while Shalistin berries are white and completely lack anthocyanins in the skin (Fig. 1A). Fig. 1B shows that the original Malian cane on the Cabernet Sauvignon vine still produces bronze coloured berries while the rest of the parental vine has dark coloured (red/black) Cabernet Sauvignon berries. Malian vines continue to exhibit some instability. For example, in 2003, several vines within a two hectare planting of approximately 3,400 vines produced white bunches, an example of which is shown in Fig. 1C. Five Malian plants bearing canes of mixed bunches (both white and bronze berries) and one plant with bronze bunches and white bunches (similar to Fig. 1C) were observed at harvest time in 2006 (Malcolm Cleggett, pers. comm.). Occasionally white sectors can be clearly seen on Malian berries (Fig. 1D). Sectors of reduced colour can also be found infrequently on individual berries from other Cabernet Sauvignon vines in the same vineyard (not shown). Shalistin has a stable white phenotype with no bunches or sectors reverting to bronze or red/black as observed on 1.6 hectares of vines (~2,500 vines) in the House vineyard, Langhorne Creek, in 2003 (personal observation) or in the previous two seasons of 2001 and 2002 (Malcolm Cleggett, pers. comm.).

Grape berries have a skin, which can be peeled from the colourless flesh. The skin (exocarp) is composed of several cell layers, some of which are coloured in red grapes. The epidermal cells, comprising only a single layer, are much smaller than the large underlying (sub-epidermal) cells of the skin and flesh. An oblique section cut through a nearly ripe Cabernet Sauvignon berry (Fig. 2A) to reveal epidermis, skin and flesh shows that the coloured cells are several layers deep extending from the epidermal layer (marked by a trapezoid shape), giving the coloured region a diffuse

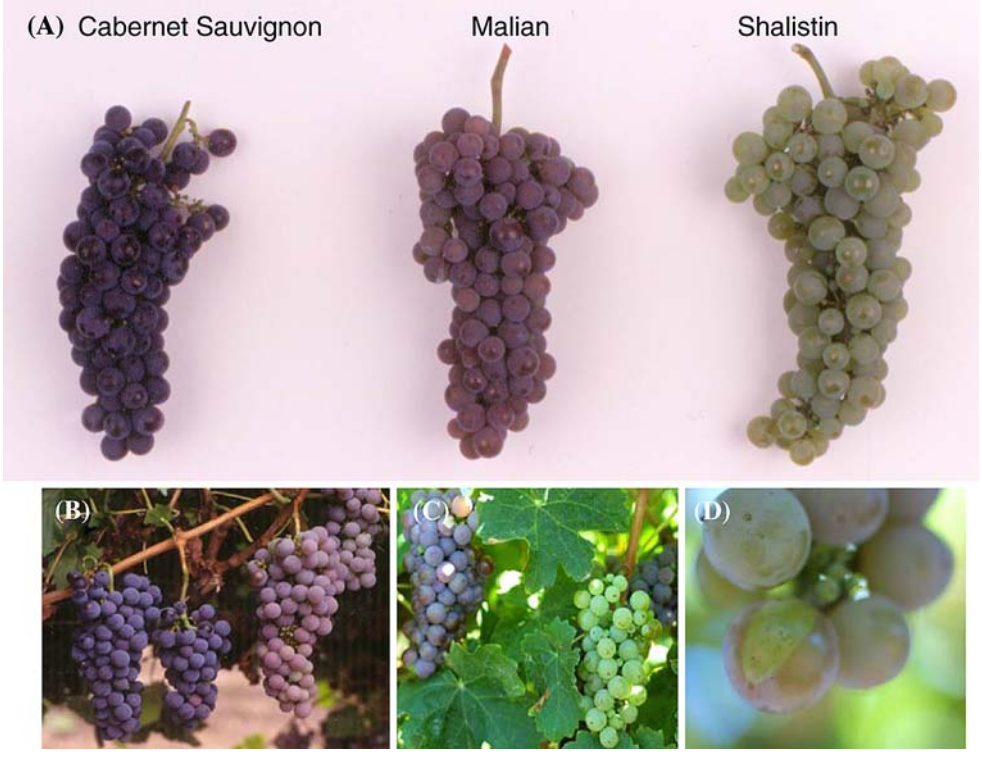

Fig. 1 Photographs of the coloured sports. (**A**) Bunches of Cabernet Sauvignon, Malian and Shalistin. (**B**) Cane bearing bronze berries on the original Cabernet Sauvignon plant. (**C**) Malian vine with white bunch. (**D**) White sector on a Malian berry

edge extending into the flesh of the berry. This is due to the presence of large cells, only some of which are coloured and two are indicated by arrows. Malian berries only contain anthocyanins in the epidermis, single cell layer appearing as a clearly defined coloured band (Fig. 2B). Shalistin sections have no red or purple colour in the skin (Fig. 2C). Light microscope examination of thin sections of a Cabernet Sauvignon berry (skin side up), after the commencement of ripening when the synthesis of anthocyanin has begun, reveals that the large subepidermal cells are coloured so that only the dark outline of the epidermal pavement cells can be seen (Fig. 2D). When Malian berry skin is viewed from above as shown in Fig. 2E, the small coloured pavement cells can be easily observed because the underlying skin cells are transparent. The coloured vacuole containing the anthocyanins is visible in many cells, the cell walls and the nucleus being colourless. Examination of Shalistin skin (Fig. 2F) confirms that the epidermal and other skin cells are not coloured in this cultivar. A diagram of cross-sections through part of a berry of each of the cultivars is shown in Fig. 2G with Cabernet Sauvignon having several layers of coloured cells constituting the skin compared to Malian with only the thin epidermis containing anthocyanins and Shalistin with a colourless skin.

The berry colour locus region

A Cabernet Sauvignon (BAC) contig was assembled containing the berry colour locus comprised of *VvMYBA* regulatory genes (Walker et al., submitted), part of which is shown in Fig. 3A. The red allele has two *MYBA* genes, *VvMYBA1* and *VvMYBA2*, both of which regulate berry colour in Cabernet Sauvignon (Walker et al., submitted). The white allele has two mutations in *VvMYBA2* disrupting protein function (Walker et al., submitted) while a retrotransposon in the promoter of *VvMYBA1* gene (Kobayashi et al. 2004) prevents transcription of the gene in berry skin; both of these genes are inactive in white alleles. Two BACs, CS20D18 and CS53P11, have distinctive restriction patterns when digested with *Hind*III (Fig. 3B) with no bands of the same size except the vector band at about 8.5 kb. Using sequence data obtained from the proximal ends of these two BACs, PCR techniques failed to identify an overlap of the BACs, though both overlapped with BAC CS16D13 (data not shown). The insert size of the two BACs was determined from the gel shown in Fig. 3C where lanes 2 and 4 show a vector band at 8.5 kb and insert bands of about 130 kb for both BACs. From this data, we can conclude that the interval between the two

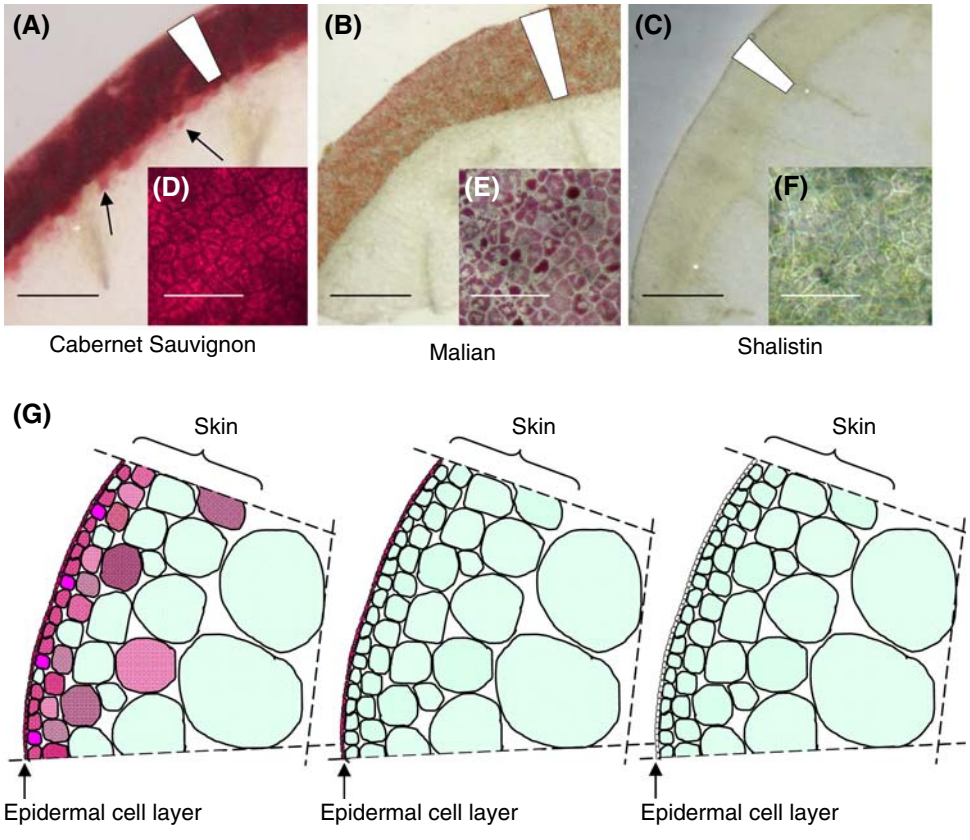

Fig. 2 Examination of berry cell layers. (**A**, **B**, **C**) Oblique angle hand sections cut on an arc through berry to show epidermis (marked with a trapezoid) and internal cells, constituting the skin and flesh. (**A**) Cabernet Sauvignon (arrows indicate large subepidermal coloured cells). (**B**) Malian. (**C**) Shalistin. (**D**, **E**, **F**) Thin surface section looking through skin (photo taken with Axioplan microscope). (**D**) Cabernet Sauvignon. (**E**) Malian. (**F**) Shalistin. (**G**) Diagrammatic representation of cross-section of Cabernet Sauvignon, Malian and Shalistin berries. Bar in A–C is 1 mm; D–F is 100 μm

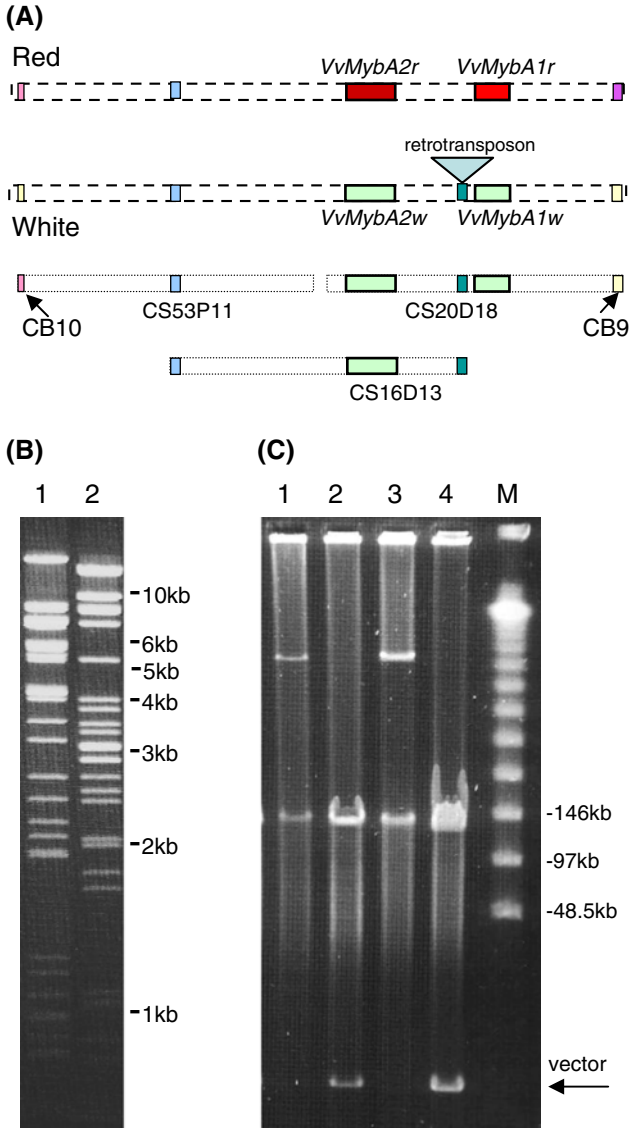

Fig. 3 Construction of a BAC contig region. (**A**) Diagram of the berry colour locus showing red and white alleles (dashed lines) and three BACs, CS20D18, CS16D13 and CS53P11 (dotted lines). Genes are marked with heavy-lined boxes and were ordered based on information from other BACs (Walker et al., submitted). BAC ends are shown as light-lined boxes. The BAC end sequences (utilised as both PCR markers and as DNA probes) used in subsequent experiments are indicated by arrows. Not to scale. (**B**) DNA extracted from BACs CS20D18 (lane 1) and CS53P11 (lane 2) restricted with *Hind*III and electrophoresed on a 0.8% TBE agarose gel. Vector band is at about 8.5 kb. (**C**) BAC DNA electrophoresed on a pulse field gel; lane 1 and 2 are BAC CS20D18; lane 3 and 4 are CS53P11; lanes 2 and 4—DNA restricted with *Not*I; lanes 1 and 3 DNA not digested; lane M—marker 48.5 kb ladder

markers (CS53P11CB10 and CS20D18CB9) derived from the distal ends of the two BACs is more than 260 kb.

Deletion of the berry colour locus in Shalistin

To determine if gross DNA alterations had occurred at the colour locus in Malian and Shalistin compared to Cabernet Sauvignon, Southern blot, PCR and sequencing experiments were performed using genomic DNA extracted from young leaves. Fig. 4A shows the map of both the white and red alleles from Cabernet Sauvignon and the relative positions of the differences between the two alleles. The two parents of Cabernet Sauvignon, the red-berried Cabernet Franc and white-berried Sauvignon Blanc (Bowers and Meredith 1997) were also included in both Southern and PCR experiments.

A 448 bp fragment of DNA from the marker 53P11CB10 (Fig. 3A and 4A) was used as a probe on a Southern blot of genomic DNA restricted with *Hind*III (Fig. 4B). Bands at 3.5 kb present in Cabernet Franc and Sauvignon Blanc are not inherited by Cabernet Sauvignon. The 6.8 kb white allele band inherited from Sauvignon Blanc is present in Cabernet Sauvignon and in both Malian and Shalistin, while the band from the red allele of 6 kb is fainter in Malian than the Cabernet Sauvignon and Cabernet Franc and is absent in Shalistin. There are no new bands visible in the Shalistin track.

A Southern blot, probed with *VvMYBA* (which hybridises to both *VvMYBA1* and *VvMYBA2* indicated in Fig. 4A), showed a band at about 5.2 kb in Cabernet Sauvignon which was inherited from Cabernet Franc (Fig. 4C). This band is lighter in Malian and absent in Shalistin. Two other bands (also arrowed) are lighter in Malian and Shalistin than in Cabernet Sauvignon despite even loading of the total genomic DNA (not shown) suggesting a deletion in Shalistin of some, but not all, of the hybridizing sequences. Other *MYBs*, with sequences sufficiently similar to *VvMYBA* to allow hybridization of the probe, might be located elsewhere in the genome and therefore appear in both Cabernet Sauvignon and Shalistin as similar bands.

VvMYBA1 and *VvMYBA2* genomic DNA fragments from Cabernet Sauvignon and Shalistin were amplified using MybAfw and MybArev primers (Table 1), separated by electrophoresis, extracted and analysed. Fig. 4D shows part of the sequence of *VvMYBA2* (sequenced using primer MybA1fw5), where there is a CA dinucleotide deletion in the white allele from Cabernet Sauvignon. Because Cabernet Sauvignon is heterozygous, both sequences are present and the red arrow shows where double sequence starts. Shalistin has only the white allele sequence so there is no double sequence observed in the chromatogram. Sequence analysis of *VvMYBA1* was also carried out

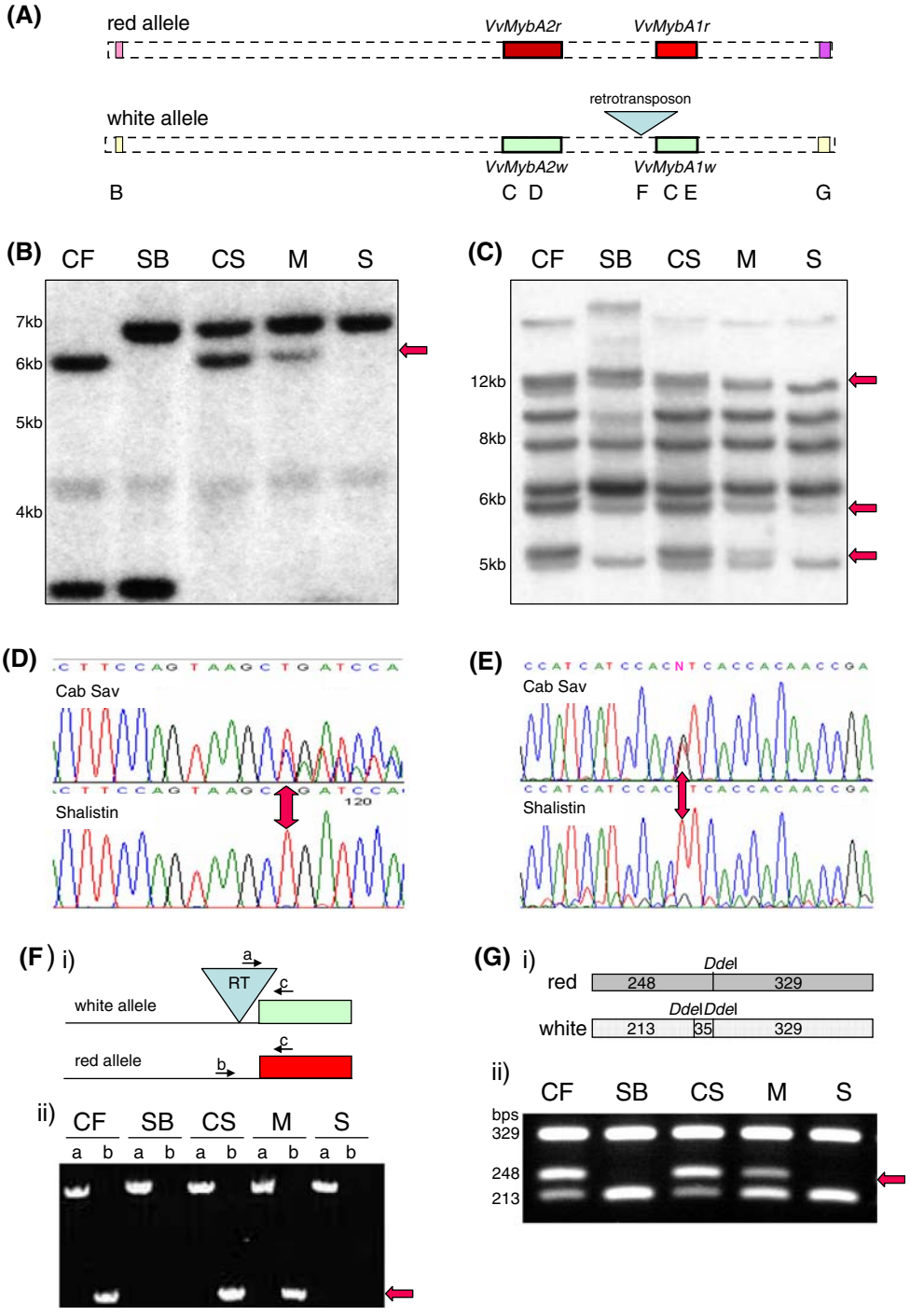

Fig. 4 Analysis of genomic DNA from Cabernet Sauvignon and its relatives in the *MYBA* region. CF—Cabernet Franc, SB—Sauvignon Blanc, CS—Cabernet Sauvignon, M—Malian, S—Shalistin. (**A**) Diagrammatic representation of the two colour alleles from Cabernet Sauvignon (not to scale). Letters under the white allele indicate the relative positions of the DNA shown in the following panels. (**B**) Southern blot of genomic DNA restricted with *Eco*RI and probed with 53P11CB10 DNA. (**C**) Southern blot of genomic DNA restricted with *Hind*III and probed with *VvMYBA1*. (**D**) Chromatogram of sequence of *VvMYBA2* using primer MybAfw5 from Cabernet Sauvignon and Shalistin. (**E**) Chromatogram of sequence of *MYBA1* using primer MybArev from Cabernet Sauvignon and Shalistin. (**F**) PCR amplified DNA using retrotransposon (RT) primers (**i**) diagram of DNA showing relative positions of primers (**ii**) cultivar DNA electrophoresed on 1.0% agarose gel. (**G**) PCR amplified DNA using primers 20D18CB9f and r, restricted with *Dde*I (**i**) diagram of DNA sequence showing restriction sites and sizes of fragments in bps (**ii**) cultivar DNA electrophoresed on 1.5% agarose gel. Red arrows indicate differences in Shalistin compared with Cabernet Sauvignon

and Fig. 4E shows an example of a single base difference between the two alleles in Cabernet Sauvignon while Shalistin has only the white version of the two sequences. As both *VvMYBA1* and *VvMYBA2* have three nucleotide differences in the coding region between the red and white alleles of Cabernet Sauvignon, the loss of the red allele sequences from Shalistin cannot be explained by single base pair mutation.

The presence of the retrotransposon in the white allele can be established by PCR (Kobayashi et al. 2004). The white allele is amplified in all five cultivars (Fig. 4Fiia) while the red allele (b) is missing from both Sauvignon Blanc and Shalistin.

PCR with the primers to the marker 20D18CB9 (Figs. 3A and 4A) and genomic DNA template resulted in a 577 bp fragment which was restricted with

*Dde*I. With red alleles a single restriction site in the fragment results in two bands of 329 and 248 bp while white alleles have an extra restriction site and result in three bands of 329, 213 and 35 bp (Fig. 4Gi). The 35 bp band is not detected on the 1.2% gel. In the Cabernet Franc and Cabernet Sauvignon lanes (Fig. 4Gii), all three bands of 329, 248 and 213 bp are seen arising from both red and white alleles, whereas in the Sauvignon Blanc lane, the white-specific 213 bp fragment is visible but the red-specific band of 248 bp is absent. In the Malian track the intensity of the red band (248 bp) has reduced compared to the Cabernet Sauvignon and Cabernet Franc tracks. The Shalistin lane contains the white band (213 bp) but not the red band (248 bp). The larger band of 329 bp is present in all tracks as would be expected. This suggests that the red allele in Cabernet Sauvignon and originally inherited from Cabernet Franc has been deleted in Shalistin while the white allele inherited from Sauvignon Blanc is still present.

In summary, red allele versions of the DNA markers 20D18CB9 and 53P11CB10 are less abundant in Malian compared to Cabernet Sauvignon and are absent in Shalistin. These data, together with results from the *MYBA* region, indicate the same genomic fragment of at least 260 kb containing the red colour allele is partially absent in Malian and completely absent in Shalistin, suggesting a large deletion of the red colour allele. Both Shalistin and Malian still contain the white allele of the colour locus.

Deletion absent in other white cultivars

In our previous paper (Walker et al., submitted) we showed that white cultivars contained a mutated version of the *VvMYBA2* gene, which encodes a truncated inactive protein, and the white version of the tightly linked CAPS marker 20D18CB9. Shalistin also has this mutant allele (Fig. 4G). However, the red allele has been deleted in Shalistin (Fig. 4). To determine if a similar deletion was present in ten other white cultivars, Southern blots were carried out using DNA from the marker 20D18CB9 as a probe. With genomic DNA restricted with *Eco*RI only a single band is detected in heterozygous cultivars like Cabernet Sauvignon showing no detectable polymorphism between red and white alleles. Fig. 5Ai shows the even loading of the gel with the exception of the last lane, Riesling (1/2) where only half the amount of DNA was deliberately loaded. In Fig. 5Aii all the hybridising bands (except the last lane) are of similar intensity and none are as weak as the last lane where half the DNA was loaded. By comparison, Shalistin has a lighter hybridising band (Fig. 5B) than other white cultivars such as Muscadelle. Another 32 white cultivars, listed in the Material and Methods section, were also tested for deletion at this locus (data not shown) but had similar hybridising intensity as the cultivars shown in Fig. 5A. This result indicated that all 42 white cultivars do not have deletions in the region of the berry colour locus and Shalistin is unique in this respect and contains a novel white allele.

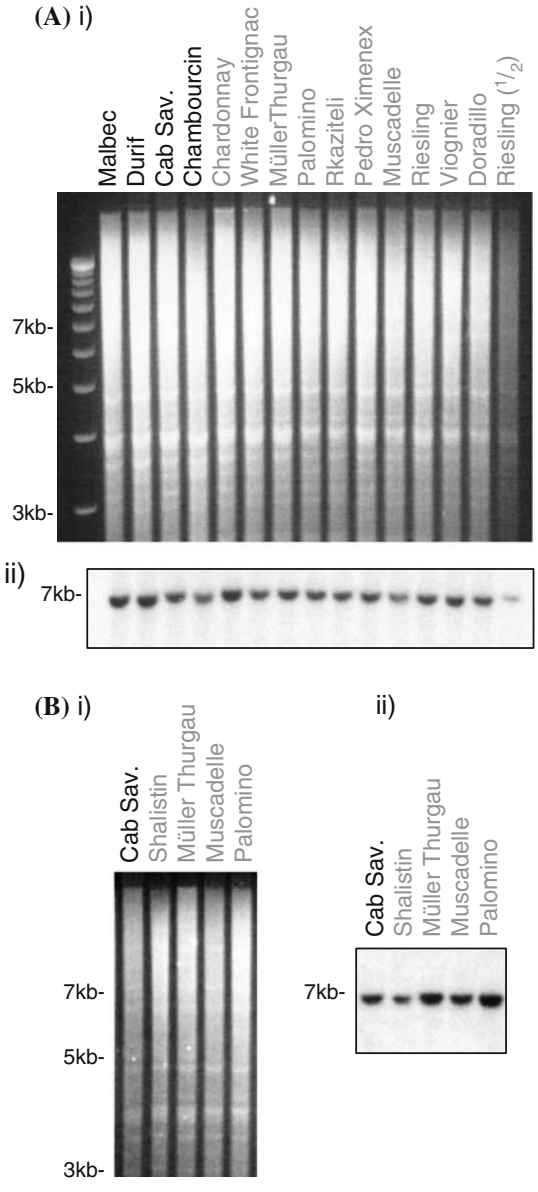

Fig. 5 Southern blots of genomic DNA from other red and white cultivars suggest both alleles are similar. (**A**) (**i**) Genomic DNA (2.5 μg) was digested with *Eco*RI, separated on a gel, blotted and (**ii**) probed with labelled 20D18CB9 DNA. The last lane (Riesling 1/2) has only 1.25 μg DNA loaded in it. (**B**) Comparison with Shalistin, (**i**) Blot prepared and (**ii**) probed as above. Red-berried cultivar names are in black and white-berried cultivars are in grey

Alterations to the colour locus in Pinot clones

Oblique sections of berries of Pinot Noir, Pinot Gris and Pinot Blanc were cut to expose both the outer surface and the flesh of the berry. Similar to Cabernet Sauvignon (Fig. 2A), Pinot Noir had coloured cells in both the epidermal and several layers of the subepidermal cells in the skin of the berry (Fig. 6A). Pinot Gris resembled Malian with coloured epidermal cells only (Fig. 6B) whereas Pinot Blanc berry cells were not coloured at all (Fig. 6C), similar to Shalistin. Thin skin sections (Fig. 6D–F) confirmed that the similarity of the colour of the cell layers between Cabernet Sauvignon and Pinot Noir, Malian and Pinot Gris, and Shalistin and Pinot Blanc.

Genomic DNA was extracted from young leaves from Pinot cultivars grown in the Coombe Vineyard and used to assess the possibility of DNA alterations in the vicinity of the colour locus in the pale-fruited cultivars. On a Southern blot, hybridisation to a *VvMYBA1* probe is reduced in some bands of Pinot Gris and particularly Pinot Blanc lanes (Fig. 6G) suggesting a deletion in the region of the *VvMYBA* genes of the red allele. Sequence analysis of PCR products from *VvMYBA1* and *VvMYBA2* of Pinot Noir and Pinot Blanc showed that Pinot Noir is heterozygous for

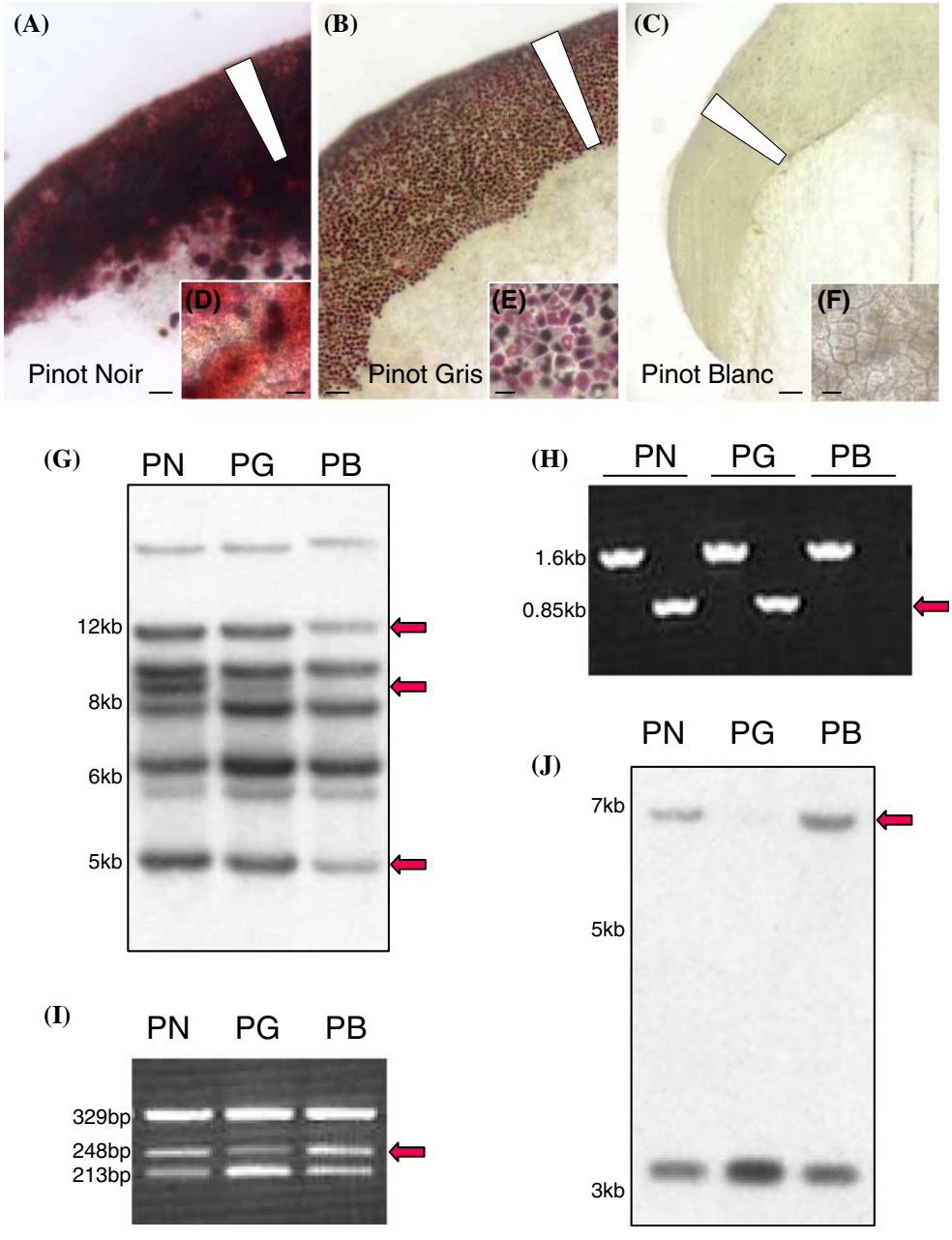

Fig. 6 Analysis of Pinot sports. PN—Pinot Noir, PG—Pinot Gris, PB—Pinot Blanc. (**A**, **B**, **C**) oblique sections through ripe berries of Pinot Noir, Pinot Gris and Pinot Blanc, respectively. White trapezoid indicates the outside surface in each section. (**D**, **E**, **F**) thin surface section looking through skin of Pinot Noir, Pinot Gris and Pinot Blanc, respectively. Pinot Noir section taken from berry just starting to colour. (**G**) Southern blot of genomic DNA, restricted with *Hind*III and hybridised with *MYBA* probe. Arrows show bands of fainter or no hybridisation in Pinot Gris and Pinot Blanc. (**H**) PCR result using retrotransposon primers shows red allele not amplified in Pinot Blanc. (**I**) CAPS marker 20D18CB9 shows a reduction in the red band in Pinot Gris but not Pinot Blanc. (**J**) Southern blot of genomic DNA restricted with *Eco*RI and probed with labelled DNA from 53P11CB10, suggesting deletion present in Pinot Gris but not Pinot Blanc. Bar in A–C is 100 μm; D–F is 20 μm. Red arrow indicates differences between Pinot Noir and either Pinot Gris or Pinot Blanc

red and white alleles in both genes while Pinot Blanc only has the white versions (data not shown). The lack of the red allele in Pinot Blanc, when amplified with the retrotransposon marker primers (Fig. 6H), supports the hypothesis that this cultivar has a deletion in the red allele in the vicinity of the *VvMYBA* genes. A single mutation such as an insertion could not be responsible for all these changes. With the CAPS marker 20D18CB9 (Fig. 6I), Pinot Blanc scores as heterozygous red, suggesting the Pinot Blanc red allele is not modified at this marker. The marker on the other side of the locus, 53P11CB10, was used as a probe for a Southern blot (Fig. 6J), and showed both Pinot Blanc and Pinot Noir have a similar strongly hybridizing band of nearly 7 kb. The presence of the red allele in Pinot Blanc at these two distant markers is different from that obtained with Shalistin. By comparison, Pinot Gris has a lighter red allele band with the marker 20D18CB9 (Fig. 6I) and hybridising band with 53P11CB10 (Fig. 6J); both of these results are similar to those of Malian (Fig. 4G and B, respectively). These results suggest the possibility of a large deletion of the *VvMYBA* region in Pinot Gris in the L2 cell layer, possibly of a large size (>260 kb) similar to the deletion in Malian.

Discussion

Similar to other plants, grapevines (Derman 1947) have at least two distinct cell layers (L1 and L2), which contribute to different parts of the plant. The L1 is responsible for the single cell layer of the epidermis, comprising stomatal complexes, trichomes (hairs) and the pavement-like cells. The L2 makes up most of the rest of the plant including the mesophyll and gametes. Mosaics and chimeras are common in the plant world (Marcotrigiano 1997) where many are periclinal chimeras (genetically different L1 and L2 cell layers), maintained through clonal propagation such as cuttings. In *Vitis vinifera*, one of the best characterised example is Pinot Meunier where a mutation in a gene involved in gibberellin perception in the L1 cell layer leads to a hairy leaf phenotype (Boss and Thomas 2002). In Pinot Meunier the chimeric cultivar has been maintained by cuttings and was finally separated into plants with each individual genotype by taking material through cultured somatic embryos (Franks et al. 2002).

Previous studies of the Pinot cépage have been studied using molecular markers and it has been established that they are in fact clonally related and genetically very similar; therefore, they have not arisen through sexual reproduction (Ye et al. 1998; Regner et al. 2000). Pinot Gris, which can display infrequent white berry sectors similar to those seen in Malian, has been identified as a periclinal chimera by analysing the phenotype of self-pollinated progeny which are derived from the L2 cell layer only (Hocquigny et al. 2004). The progeny only had white berries suggesting a mutation preventing anthocyanin synthesis was present in the L2 layer in the parent, Pinot Gris. Malian and Shalistin are new grape cultivars, which have arisen as buds sports of Cabernet Sauvignon (Cleggett 2002; 2003). The colour phenotype of the berries from Malian resembles Pinot Gris while Shalistin is similar to Pinot Blanc in having no anthocyanin in the skin, leading to the hypothesis that Malian might also be a periclinal chimera.

Observations of sections through nearly ripe berries show that Malian has only a single cell layer, the epidermis, which contains cells with anthocyanins present. This is in contrast to Cabernet Sauvignon (shown in this report) and other red grape cultivars (Barceló et al. 1994) where several cell layers beneath the epidermis also contain anthocyanin. The results of these observations support the suggestion that Malian is a periclinal chimera with the L1 cell layer capable of making anthocyanin whilst the underlying cell layers (L2) cannot. Sometimes Malian also gives rise to anticlinal chimeric material where both cell layers of part of the meristem have been altered producing a cane with white fruit or a bunch with both bronze and white fruit (a genetic mosaic phenotype).

Malian exhibits some instability and can produce canes with white fruit at a frequency of about 1–2 per thousand vines per year. Several plants in a 1.6 Ha vineyard have bunches with white berries in any year (Mac Cleggett, pers.comm.), which suggests a genetic instability much higher than the reversion rate of Shalistan mutating back to red. By comparison, Pinot Noir vines were examined for mutation to white fruit (Müller-Stoll 1950); however, no changes of this type were found. Since two alterations would be required to convert Pinot Noir berries to white berries, it is not surprising that white berries were not observed.

The berry colour locus of Cabernet Sauvignon is composed of two very similar and adjacent genes, either of which could control berry colour (Walker et al., submitted). As the red allele of Cabernet Sauvignon contains the functional forms of both genes, mutation in a single gene, such as a base change or the insertion of a transposable element, would not alter the phenotype of the plant, even if both cell layers contained the mutation. Inactivation of both *VvMYBA1* and *VvMYBA2* genes by mutation or deletion in the red allele of a heterozygous plant are necessary to

produce the phenotypes seen in Malian and Shalistin. There are several types of mutation that could result in two inactive genes and include inversion of the DNA with the breakpoints in the genes, insertion of a transposable element, which affects both genes, or insertion of an element and subsequent rearrangement of the DNA. Somatic recombination of the region so that Shalistin has two copies of the white allele is also possible but based on the Southern analysis of the region in Figs. 4B, C and 5 this is unlikely. Based on sequence, marker and southern analysis, our results clearly demonstrate that deletion of a large region including both *VvMYBA1* and *VvMYBA2* genes is the most likely cause of the alteration in phenotype seen in Malian and Shalistin.

A model is presented in Fig. 7 for the way in which the Cabernet Sauvignon bud sports have arisen. It has been proposed that *Vitis* plants have only two cell layers (Thompson and Olmo 1963). In Cabernet Sauvignon plants, both the L1 and L2 layers are heterozygous for the berry colour locus giving rise to a coloured epidermis derived from the L1 layer and several sub-epidermal coloured layers in the skin derived from L2, shown in Fig. 2A. A deletion event occurs in a meristematic cell in the L2 cell layer leading in the loss of at least 260 kb of DNA of the red version at the berry colour locus. This mutated cell would be the progenitor of all L2 cells in that cane or branch of the vine. All berries along that cane would then have the Malian phenotype of bronze coloured berries, in which anthocyanin is no longer present in the sub-epidermal cells. The deletion could be the result of a recombination event between two sequences, which flank the berry colour locus (Schuermann et al. 2005; Tuteja et al. 2001). The chimera would be generally maintained but occasionally cells from the L2 could invade the L1 cell layer as a result of damage either by insects or mechanical disruption. The differentiation of plant cells is based on their position in the meristem and not their clonal origins so the invading cells from L2 would take on the role of L1 cells and go on to form the epidermis (Kidner et al. 2000). In Shalistin, the L2 cells with the deletion have become incorporated in the L1 cells in the meristem so both epidermal and sub-epidermal cells have the deletion of the red allele of the colour locus and can no longer make coloured berries (Fig. 7). Once the deletion is established in both cell layers, reversion to bronze or red cannot occur through an alteration in cell position because the red berry colour allele has been deleted from this cultivar. Our data supports this theory with the molecular lesion in L2 being a large deletion, which includes both red alleles of the *VvMYBA1* and *VvMYBA2* genes of the berry colour locus.

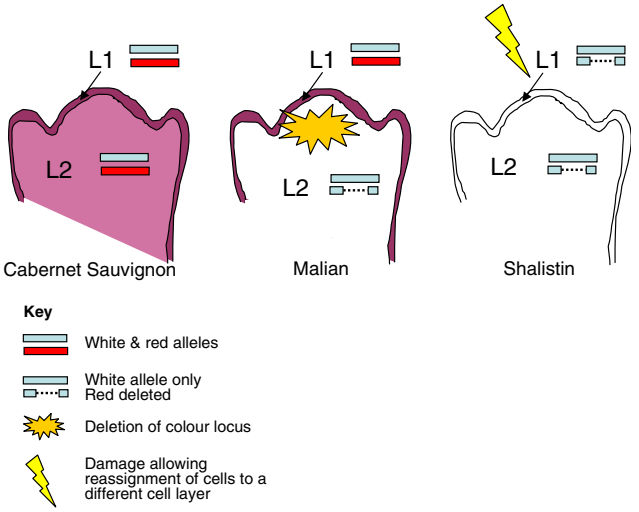

Fig. 7 Model of the hypothesis of the formation of the Cabernet Sauvignon sports. The two cell layers in the meristem with two organ primordia are shown as coloured and containing the red allele in Cabernet Sauvignon. The genotype of each cell layer is represented by coloured bars. A deletion event occurs in the L2 giving rise to Malian. L2 cells from Malian replace the L1 cells in Shalistin resulting in complete deletion of the red allele in this sport

Microsatellite analysis has been used to compare available clones of Pinot Gris (65) and Pinot Blanc (30) with Pinot Noir and Pinot Meunier clones (Hocquigny et al. 2004) and have been grouped depending on which alleles of the microsatellites are present. The results indicated that the Pinots are all clonally related but that there are groups of Pinot Gris and Pinot Blanc clones which have as many as three differences in the 50 markers tested. Since the Pinot cépage is an ancient family possibly going back to Roman times (Hocquigny et al. 2004), clones have had the opportunity to accumulate somatic mutations, some of which lead to phenotypic changes as seen with the Pinot colour sports and Pinot Meunier. The colour sports are thought to have arisen on a number of occasions so it is possible that different types of mutations could have occurred. Deletions of different sizes of the red berry colour allele removing both active red allele genes are possible mechanisms. We have demonstrated here that a large deletion in Shalistin is the probable cause of the change in phenotype, whereas a similar large deletion has not occurred to give rise to Pinot Blanc. Pinot Blanc could have a small deletion removing the *MYBA* genes but not the flanking markers. Alternatively, insertion of transposable elements into both *MYBA* genes in Pinot Blanc could give a similar result but seems a less likely scenario. Our data suggest that the Pinot Blanc clone in the Coombe Vineyard has not

arisen from the Pinot Gris clone planted in the same vineyard, as the changes in each are not similar. Pinot Gris has differences in both 20D18CB9 and 53P11CB10 compared to Pinot Noir whereas Pinot Blanc is similar to Pinot Noir with both markers. Pinot Gris could have a large deletion of the colour locus or it could be the result of somatic recombination in the region so that Pinot Gris has two copies of the white allele in the L2 cell layer. Without a white bud sport from this clone, it is difficult to determine the exact nature of the mutation. However, the available Pinot Blanc clone is unlikely to have come from the clone of Pinot Gris that we used in these experiments.

Consequentially, the sports of Cabernet Sauvignon, where a documented series of phenotypic alterations has occurred, are extremely valuable in understanding the changes that can take place to produce new cultivars.

Most plants have three identifiable cell layers. The L3 contributes to the vascular tissue and some central tissues of the leaf and stem but not to the parts required for sexual reproduction. Determination of the number of cell layers is usually carried out by microscopic examination of the planes of cell division in the plant meristem. The meristematic region is very small and results of sectioning could be difficult to interpret accurately. If *Vitis* plants do have three cell layers as suggested by leaf variegation observations (Derman 1947), our model for how Shalistin has arisen would be very similar to that proposed in Fig. 7. The deletion might occur in the L3 cell layer of Cabernet Sauvignon but no phenotypic difference would be apparent. An alteration in phenotype would only be detected when the mutant L3 cells invade the L2 layer and become established as L2 cells, and subsequently invade the L1 by L2 cells as indicated in Fig. 7.

The origin of many grape cultivars is not known because they have been collected over several thousand years. Other white cultivars, which were examined by Southern analysis, do not show a deletion of the locus suggesting that all have been the result of a sexual propagation in which the parents each provided a white berry colour allele, making all these cultivars homozygous white at the berry colour locus (Walker et al., submitted).

On very rare occasions, some white berried cultivars have produced red berried bud sports. For example, Chardonnay and Sultana have both produced bud sports called Red Chardonnay and Pink Sultana, respectively (Boss et al. 1996). Kobayashi et al. (2004) studied one red revertant, Ruby Okuyama, and showed that recombination, removing most of a retrotransposon from the promoter of the white *VvMYBA1* gene, allowed transcription of the gene and subsequently the reversion in phenotype. It is possible that other white-to-red sports such as Red Chardonnay have evolved by excision of the retrotransposon and this type of event could occur at a low frequency in any white cultivar. This is different from and independent of the deletion mechanism, elucidated in this report, by which white bud sports arise on red cultivars.

In grapes, berry colour is controlled by two similar *MYB* regulatory genes, either of which would be sufficient to produce colour in the fruit (Walker et al., submitted). Most white cultivars have come from sexual reproduction of a progenitor in which both genes were inactivated by different mechanisms of mutation and insertion of a retrotransposon. However, some cultivars such as Malian and Pinot Gris are chimeras, resulting from somatic mutation at the berry colour locus.

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