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Chimerism in grapevines: implications for cultivar identity, ancestry and genetic improvement

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Abstract In the course of DNA profiling of grapevine cultivars using microsatellite loci we have occasionally observed more than two alleles at a locus in some individuals and have identified periclinal chimerism as the source of such anomalies. This phenomenon in long-lived clonally propagated crops, such as grapevine, which contains historically ancient cultivars, may have a role in clonal differences and affect cultivar identification and pedigree analysis. Here we show that when the two cell layers of a periclinal chimera, Pinot Meunier, are separated by passage through somatic embryogenesis the regenerated plants not only have distinct DNA profiles which are different from those of the parent plant but also have novel phenotypes. Recovery of these phenotypes indicates that additional genetic differences can exist between the two cell layers and that the Pinot Meunier phenotype is due to the interaction of genetically distinct cell layers. It appears that grapevine chimerism can not only modify phenotype but can also impact on grapevine improvement as both genetic transformation and conventional breeding strategies separate mutations in the L1 and L2 cell layers.

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Introduction

Grapevine is a clonally propagated crop and reproducible grapevine cultivar identification using microsatellite DNA profiles is possible because vegetative propagation fixes the genetic complement of the cultivars which are highly heterozygous (Thomas and Scott 1993). Regardless of the genetic stability associated with vegetative propagation, it has still been possible for "within" cultivar improvement to proceed slowly by a process known as clonal selection where superior individuals (clones) are identified in a specific environment and subsequently propagated. The mechanisms responsible for grapevine clonal differences may include changes in disease (e.g. virus) load, epigenetic differences, genetic mutation, or various combinations of these effects. Such selections most commonly retain the original cultivar name and acquire an identifying clone name. Thus cultivar improvement by this means is especially valuable for winegrape cultivars because industry and market forces and, in some instances, governmental controls discourage the adoption of new varieties from breeding programs. Recent advances in grapevine transgenic technology, which does not involve a breeding step, have the potential to have a significant impact on the improvement of established cultivars if transgenic plants are essentially unchanged from the original cultivar apart from the introduced transgene.

Vitis vinifera, the major cultivated grapevine, was domesticated around 7,000–6,000 BC (Eynard and Dalmasso 1990), and while different cultivar names number 14,000 to 24,000 (Alleweldt 1988; Viala and Vermorel 1909) actual cultivar numbers have been estimated to be between only 5,000 and 8,000 (Alleweldt 1988). Sequence-tagged microsatellite sites or simple sequence repeat (SSR) DNA markers have proved suitable for consolidating grapevine cultivar identification (Botta

et al. 1995; Bowers et al. 1996; Grando and Frisinghelli 1998; Loureiro et al. 1998; Thomas and Scott 1993; Thomas et al. 1994; Vignani et al. 1996) and investigating the pedigree of cultivars (Bowers et al. 1999; Bowers and Meredith 1997; Sefc et al. 1997, 1998; Thomas and Scott 1993; Thomas et al. 1994).

Microsatellite loci mutation rates of $>3.1\times10^{-8}$ to 4.5×10^{-9} mutations/cell/generation have been reported for normal human somatic cells (Boyer and Farber 1998) and rates between 4.5×10^{-4} and 1.6×10^{-6} mutations/gamete per locus have been estimated to occur during meiosis (Edwards et al. 1992; Kwiatkowski et al. 1992). It is thought that DNA polymerase slippage is the most likely cause of mutations resulting in the length variation of microsatellite repeats (Schlotterer and Tautz 1992), and it has previously been suggested that a microsatellite mutation during meiosis may have been responsible for difficulty with assigning the likely pedigree of a grapevine cultivar (Thomas et al. 1994).

Due to continuous clonal propagation of grapevine cultivars and phenotypic evidence for the occurrence of somatic mutations, for example differences in berry colour of Pinot clones (Viala and Vermorel 1909), there is a reasonable likelihood that somatic mutations have also occurred at microsatellite loci within the grapevine genome. Indeed, in the course of typing grapevine varieties and cultivars, and clonal selections of these, we have identified three instances of somatic mutations associated with microsatellite loci due to the appearance of triple-allele genotypes. Here we show for one such case that chimerism in the cell layers of the grapevine shoot can account for the maintenance of three alleles at a locus in an individual. We go on to describe the complete separation of the genotypically and phenotypically distinct cell layers of a grapevine chimera by passage through somatic embryogenesis. These observations lead to a discussion of the implications that grapevine chimerism has for cultivar improvement as well as the application of DNA-profile analysis for determining cultivar identity and ancestry.

Material and methods

Source of plant material

Leaf tissue for DNA extraction was collected from individual plants growing in vineyards in Australia, France and Italy (see Tables 1 and 2).

DNA extraction and DNA profiles

Large-scale DNA extraction was as previously described (Thomas et al. 1993). For small-scale extraction of DNA from in vitro leaves of plants regenerated from somatic embryos about 50 mg of tissue was ground in liquid nitrogen in a 2-ml microfuge tube using a disposable pestle. The ground tissue was re-suspended in 500 µl of buffer A (Thomas et al. 1993) and left for 2 min before centrifugation for 4 min. The supernatant was discarded and the pellet was re-suspended in 500 µl of DNAzol reagent (Gibco-BRL) and left for 1 min before microfuging for 10 min. The supernatant was transferred to a new 1.5-ml microfuge tube and re-centrifuged for 5 min. Ethanol (250 µl) was added to the supernatant and left at room temperature for 3 min. The DNA was pelleted by microfuging for 10 min, washed with 70% ethanol and briefly vacuum dried before re-suspension in 200 µl of TE. Following one phenol/chloroform and one chloroform extraction, the DNA was re-precipitated with ethanol and re-suspended in TE (30 µl).

PCR primer pairs were VVS1, VVS2, VVS5, VVS16, VVS29, VVS19 (Thomas et al. 1994) and VVMD7 (Bowers et al. 1996), and conditions for PCR of grapevine STS sites and GENESCAN analysis of PCR products have been described previously (Thomas et al. 1994).

Cloning and sequencing of PCR products

DNA sequence information was obtained at the Pinot Meunier VVS2 locus by cloning PCR allele fragments isolated from a non-denaturing TAE-buffered polyacrylamide gel into a T-tailed pBluescript SK+ vector, prior to automated sequence analysis (ABI 373).

Initiation of embryogenic culture and regeneration of plantlets

Embryogenic callus was initiated from the filaments of anthers collected from a Pinot Meunier plant (clone H10V5 8099) that was growing in the Waite Agricultural Research Institute vineyard at Urrbrae, South Australia, and hence easily accessible for experimentation. Embryogenic cultures were initiated as described previously (Franks et al. 1998) except that the callus initiation medium was : major elements (Nitsch and Nitsch 1969), minor elements, Fe-EDTA (Murashige and Skoog 1962), vitamins (Gamborg et al. 1968), 60 g/l of sucrose, 2.5 µM of 2,4-D (2,4-dichlorophenoxyacetic acid), 5.0 µM of 4CPPU [N-(2-chloro-4-pyridyl)-n′-phenylurea], 2.5 µM of NOA (2-napthoxyacetic acid), and 0.3% phytagel (Sigma), pH 5.7. The embryogenic callus which appeared was transferred to a medium (GS1CA) which favoured the formation and proliferation of somatic embryos involving: major elements (Nitsch and Nitsch 1969); minor elements, Fe-EDTA (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 60 g/l of sucrose, 10.0 µM of NOA, 1.0 µM of BAP (6-benzylaminopurine), 20.0 µM of IAA (indole-3-acetic acid), 1% bactoagar, and 0.25% activated charcoal, pH 6.2. Cultures were grown in the dark at 28°C and embryos were transferred to GS1CA media without hormones to encourage germination. Germinated embryos were transferred to plant growth media [major and minor elements, Fe-EDTA; (Murashige and Skoog 1962), B5 vitamins; (Gamborg et al. 1968), 1.5% sucrose, 1% bactoagar, pH 5.7] plus 10.0 µM of BAP, in the light, to encourage shoot formation. Once a growing tip emerged it was transferred to plant growth media containing 0.5 µM of NAA (∂-napthaleneacetic acid) until roots formed. Plantlets were then transferred to soil and allowed to acclimatise slowly in a growth room for about 1 week before transfer to a controlled environment chamber and then to a glasshouse.

Root-tip chromosome counts

Root-tips were collected from potted plants and transferred to water at 4°C for 20 h. After cold-water treatment the root-tips were fixed in methanol:glacial acetic acid (3:1) for 1 h at 4°C. Root-tips were then washed three times with 70% ethanol and transferred to acid-carmine stain (Snow 1963) where they stayed at 4°C for at least 72 h before being rinsed in 70% ethanol and squashed in 45% acetic acid.

Results

Genotypic analysis of Primitivo and Pinot selections

Analysis of six plants of Primitivo at seven microsatellite loci found identical DNA profiles for all except one, Primitivo di Gioia (Table 1). In this case three alleles (164, 186, 188 bp) instead of the usual two (164, 186 bp) were observed at the VVS19 locus. The same DNA profile was obtained in the following year for new growth from the pruned vine, indicating stability of the genotype.

Microsatellite somatic mutations were also detected in DNA profiles of Pinot plants (Table 2). The genotype at the VVS2 locus for each of four Pinot Meunier plants differed from that observed for the plants of Pinot noir, Pinot gris and Pinot blanc. All of the tested Pinot Meunier plants had three alleles (129, 138, 153 bp) at the locus whereas the other Pinot plants had only two alleles (138, 153 bp) at the locus. Furthermore, two Pinot Meunier plants showed an additional mutation associated with the VVS5 locus. Once more, three alleles (121, 123, 148 bp) were observed instead of the expected two alleles (121, 148 bp) present in the other Pinot Meunier and Pinot plants. PCR fragments representing the three microsatellite VVS2 alleles were amplified from a Pinot Meunier plant and sequenced (Fig. 1). Common sequences in the flanking regions of the amplified fragments indicated that the three microsatellites are allelic. The differences in size of the three alleles were found to

Fig. 1 Aligned partial sequence of the three microsatellite alleles at the VVS2 locus which were amplified by PCR from Pinot Meunier. For clarity of presentation only part of the flanking sequence surrounding the microsatellite is presented. All microsatellite allele flanking sequences are identical to the original VVS2 sequence (GeneBank: G64021; dbSTS: 94888)

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            1         11         21         31         41         51         61         71         81         91
            |---------|---------|---------|---------|---------|---------|---------|---------|---------|-----
allele  1   CCGCAAAGCTGGAGAATATTTCATATAATGCAAGATAAAGACCATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTTGAAAC
allele  2   CCGCAAAGCTGGAGAATATTTCATATAATGCAAGATAAAGACCA--------------TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTTTGAAAC
allele  3   CCGCAAAGCTGGAGAATATTTCATATAATGCAAGATAAAGACCA----------------------TCTCTCTCTCTCTCTCTCTCTTTTTGAAAC
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Table 1 DNA profiles of Primitivo plants

Plant[a]	Location/origin[b]	Size of alleles at STS loci (base pairs)						
		VVS19	VVS1	VVS2	VVS29	VVS5	VVS16	VVMD7
Primitivo	France/France	164:186	188:190	134:144	171:179	148:–	286:–	247:249
Primitivo	France/France	164:186	188:190	134:144	171:179	148:–	286:–	247:249
Primitivo	Italy/Italy	164:186	188:190	134:144	171:179	148:–	286:–	247:249
Primitivo di Gioia	Australia/US	164:186:188	188:190	134:144	171:179	148:–	286:–	247:249
Zinfandel	Australia/US	164:186	188:190	134:144	171:179	148:–	286:–	247:249
Zinfandel	Australia/US	164:186	188:190	134:144	171:179	148:–	286:–	247:249

[a] Different names are synonyms for the cultivar Primitivo

[b] Location plant is grown at the country indicated, whereas the origin of the plant is traced back as far as possible from limited germplasm records. US=United States

Table 2 DNA profiles of Pinot plants

Plant[a]	Location/origin[b]	Size of alleles at STS loci (base pairs)					
		VVS2	VVS1	VVS29	VVS5	VVS16	VVMD7
Pinot Meunier	Australia/US	129:138:153	183:190	171:179	121:148	286:–	239:243
Pinot Meunier	Australia/NZ	129:138:153	183:190	171:179	121:123:148	286:–	239:243
Pinot Meunier	Italy/Italy	129:138:153	183:190	171:179	121:123:148	286:–	239:243
Pinot Meunier	Australia/Australia	129:138:153	183:190	171:179	121:148	286:–	239:243
Pinot blanc	Australia/Australia	138:153	183:190	171:179	121:148	286:–	239:243
Pinot blanc	Italy/Italy	138:153	183:190	171:179	121:148	286:–	239:243
Pinot blanc	Italy/Italy	138:153	183:190	171:179	121:148	286:–	239:243
Pinot gris	Italy/Italy	138:153	183:190	171:179	121:148	286:–	239:243
Pinot gris	Italy/Italy	138:153	183:190	171:179	121:148	286:–	239:243
Pinot noir	Australia/US	138:153	183:190	171:179	121:148	286:–	239:243
Pinot noir	Australia/US	138:153	183:190	171:179	121:148	286:–	239:243
Pinot noir	Italy/Italy	138:153	183:190	171:179	121:148	286:–	239:243

[a] Different names are clone names for the cultivar Pinot and identify the colour of berries or leaf hairiness

[b] Location plant is grown at the country indicated, whereas the origin of the plant was traced back as far as possible from limited germplasm records. US=United States, NZ=New Zealand

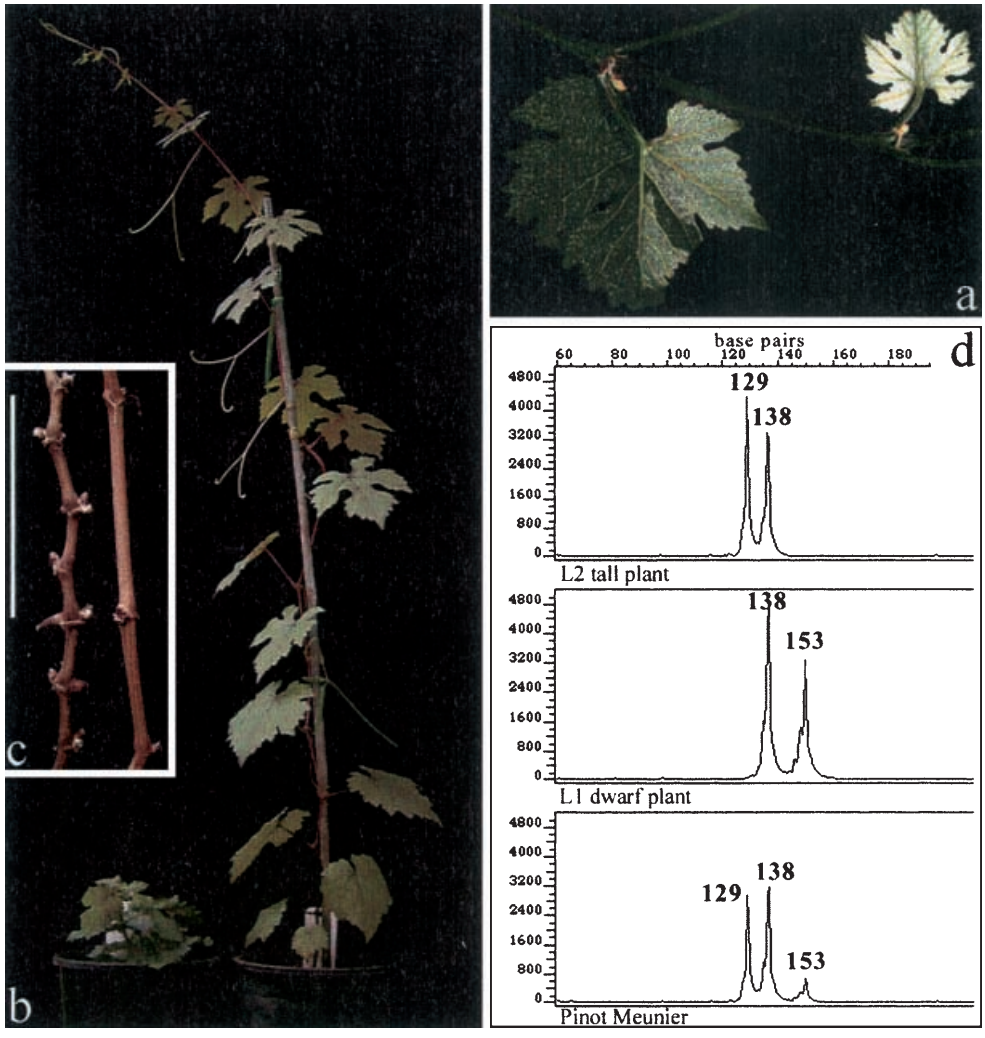

Fig. 2a–d **a** A shoot of Pinot Meunier showing hairless sectors. The smallest leaf is covered completely with a dense white mass of hairs. On the largest leaf, dense hair-covered sectors are interrupted by two hairless sectors where the L2 cell layer has displaced the L1 cell layer. **b** Plants representing the two phenotypic classes which were regenerated from the embryogenic culture of Meunier. The dwarf type (L1) is shown on the left and a Pinot noir-like (L2) type is on the right. The plants were the same age. **c** Bare stems from plants of the two phenotypic classes showing differences in internodal lengths. Dwarf is left and Pinot noir-like is right. Bar=6 cm. **d** Representative (GENESCAN) DNA profiles for the VVS2 SSR locus of Pinot Meunier (bottom) and plants from the two phenotypic classes, L1 dwarf (middle) and L2 Pinot noir-like (top)

Table 3 VVS2 DNA profiles of Pinot, Meunier and plants regenerated from somatic embryos of Pinot Meunier

Plant	Phenotype[a]	VVS2 locus alleles (bp)
Pinot noir	Hairless leaves	138:153
Pinot Meunier	Hairless leaf section (L2)	129:138
Pinot Meunier	Hairy leaves	129:138:153
Regenerated plant 1	L2	129:138
Regenerated plant 2	L1	138:153
Regenerated plant 3	L1	138:153
Regenerated plant 4	L1	138:153
Regenerated plant 5	L1	138:153
Regenerated plant 6	L2	129:138
Regenerated plant 7	L1	138:153
Regenerated plant 8	L1	138:153
Regenerated plant 9	L1	138:153

[a] L1=dwarf plant, short internodes, small hairy leaves; L2=normal tall plant, long internodes, large hairless leaves

be due to length differences in the TC dinucleotide repeat region and not due to other mutations.

In Pinot Meunier vineyards one can, on rare occasions, observe a leaf which displays a sectored appearance where part of the leaf lacks the normal hairy phenotype as a result of the L2 cell layer replacing the L1 cell layer (Fig. 2a). The genotype at the VVS2 locus of DNA extracted from sectors of Pinot Meunier leaf tissue lacking the typical hairy phenotype (Fig. 2a), where the L2 cell layer had replaced the L1 cell layer, consisted of only two alleles (129, 138 bp; Table 3).

Phenotypic and genotypic analysis of regenerants from Pinot Meunier embryogenic culture

Plants regenerated from Pinot Meunier somatic embryos exhibited one of two distinct phenotypes. Seven plants

were short due to short internodal segments and had small hairy leaves, whereas two plants were tall and had long internodal segments with normal size leaves that were relatively glabrous (Fig. 2b, c). These phenotypes are different to Pinot Meunier which has a tall habit with normal size leaves covered with a dense white mat of hairs. Both regenerated plant types had the expected diploid chromosome number (2n=38; data not shown).

Analysis of alleles at the VVS2 locus showed that all of the plants regenerated from the somatic embryo culture of Pinot Meunier had only two of the three alleles which are present in Pinot Meunier (Fig. 2d). The plants fell into two genotypic classes which corresponded to the two phenotypic classes (Table 3). For each of the seven dwarf, small-leaved plants only the 138-bp and 153-bp alleles were amplified. Whereas only the 129-bp and 138-bp alleles were amplified from DNA extracted from the two tall, large-leaved plants. The DNA profiles for five other microsatellite loci (VVS1, VVS2, VVS5, VVS29, VVS16 and VVMD7) were determined for one representative plant from each phenotypic class. For each of the five loci the DNA profile was the same as those for the Pinot Meunier clonal selection from which the embryogenic culture was established (data not shown).

The microsatellite data in Table 3 show that the dwarf plants are derived from the L1 (outer) cell layer of Pinot Meunier and the tall plants from the L2 (inner) cell layer. The L1-derived dwarf plants had a different phenotype to Pinot Meunier and Pinot noir, a different DNA profile to Pinot Meunier, but the same DNA profile as Pinot noir. The DNA profile of the L2-derived plants was different to that of both Pinot Meunier and Pinot noir, but the plants regenerated from this cell layer had the Pinot noir phenotype apart from leaf lobation which is a juvenility trait as a result of regeneration by somatic embryogenesis (Franks et al. 1998).

Discussion

Origin of somatic mutations in Pinot and Primitivo

Pinot was described by Columella in the first century AD and is thought to be a least 2,000 years old and of French origin. Benedictine monks are believed to have brought Primitivo to Italy in the 17th century. Germplasm records from Australia, Italy, USA and New Zealand were examined in an effort to try and determine the original European source and the relative age of the microsatellite mutations in Primitivo and Pinot Meunier, but the oldest records dated back only to 1960. However the presence of the VVS2 microsatellite mutation in all Pinot Meunier clones tested suggests that the mutation is very old and may have arose in Pinot either before, or shortly after, the mutation associated with the hairy leaf phenotype. Evidently the VVS5 mutation occurred later. The presence of the VVS19 microsatellite mutation in a single Primitivo clone suggests that this mutation may be relatively recent. However, what can not be discounted is that the vagaries involved in the choice of propagation material by humans may impose chance selection on neutral microsatellite mutations and determine which become common, scarce or extinct within a particular cultivar.

Phenotypic and genetic markers differentiate Pinot Meunier cell layers

In grapevine, the shoot apical meristem is considered to be composed of only two (L1 and L2) distinct cell layers (Thompson and Olmo 1963). The most-likely interpretation of the presence of three microsatellite alleles per locus in Primitivo di Gioia and Pinot Meunier is that one of the two (diploid) alleles has mutated and, through vegetative propagation, this mutation has been maintained in one of the distinct cell layers of the apical meristem while the original genotype is maintained in the other cell layer of the plant (i.e. as for a periclinal chimera). The effective genotype will therefore be derived from two cell layers. While one of the alleles will occur in both cell layers, the mutated allele will differ between the two layers and there will be effectively three different alleles present in the whole plant.

Confirmation of this interpretation was obtained for Pinot Meunier by using the phenotypic differences between the cell layers to identify and DNA-type one individual cell layer, and DNA-profile individual plants regenerated from the two different cell layers. The shoot tips and leaves of field-grown Pinot Meunier are covered in a dense white mat of hairs (Galet 1979) but occasionally a leaf will exhibit hairless sectors in which cells from the L2 layer displace cells in the L1 (epidermal) layer (Fig. 2a). In vitro plant regeneration from a fragmented shoot-tip culture of Pinot Meunier appears to increase the rate of re-organisation of the two cells layers and the appearance of sectored leaves on regenerated plants (Skene and Barlass 1983). Using the VVS2 microsatellite marker, we found that the phenotypically distinct cell layers of Pinot Meunier were genetically distinguishable (Table 3).

Somatic embryogenesis of Pinot Meunier uncovers novel genotypes and phenotypes

Plants regenerated from an embryogenic culture of Pinot Meunier were derived from cells originating from either the L1 or L2 cell layers, based on their classification into two groups according to their phenotype and genotype. Dwarf plants had the same VVS2 genotype of Pinot (138, 153 bp) whereas tall plants had a unique VVS2 genotype (129, 138 bp) as a result of being derived from the L2 cell layer of Pinot Meunier.

The efficient separation of the cell layers of a chimera by passage through somatic embryogenesis provides genetic confirmation of histological studies (Krul and Worley 1977; Faure et al. 1996) that somatic embryos of

Vitis develop from single cells. It also demonstrates that both L1 and L2 cell layers of the anther filament are competent to form an embryogenic callus.

The observation that the phenotype of the two classes of regenerated plants are different from each other and the parent Pinot Meunier plant suggests that the distinct Pinot Meunier phenotype is the result of an interaction between the genetically different L1 and L2 layers. It also appears likely that both altered plant stature in L1 plants and altered trichome formation in Pinot Meunier are associated with the same single mutation in the L1 layer.

Implications of chimerism for cultivar identity and ancestry

The hairy character in the L1 layer is not associated with the mutated microsatellite allele (129 bp) in the L2 layer. Due to the low-likelihood that non-L2 layers contribute genetic material to seed progeny (Marcotrigiano and Bernatzky 1995), the effective genotype of a chimeric plant such as Pinot Meunier during breeding will be that of cells in the L2 layer. In this case the 129-bp microsatellite mutation will be passed on to 50% of progeny, but the mutation in L1 associated with dense hairs is likely to be lost. This has relevance to studies investigating cultivars of unknown parentage where discrepancies are present in DNA profiles or phenotype because the effective genotype of the whole parent plant may not correspond to the genotype available for reduction to gametes. For example, the mutated allele (129 bp) identified in this study is the same size as the allele present in the variety Romorantin and provides one explanation for the origin of the unique microsatellite genotype of Romorantin, a putative progeny of Pinot noir, identified in a recent parentage study (Bowers et al. 1999). Thus it is possible that Romorantin may be the progeny of Pinot Meunier and not Pinot noir.

Another very recent study, based on microsatellite profiles, concluded that Schwarzriesling (a synonym for Pinot Meunier) is a likely parent of Pinot (Regner et al. 2000a). However, the study missed the presence of the three alleles at the VVS2 locus of Pinot Meunier and did not consider the possibility that Pinot may be prone to accumulate mutations (see below). Re-interpretation of their data based on the results presented in our study supports the view that Pinot Meunier is a clone, and not a parent, of Pinot. This highlights the importance of extracting DNA from tissue representative of both the L1 and L2 cell layers, and the use of sensitive techniques for microsatellite allele detection and careful scrutiny of minor peaks/bands as it is often easy to miss instances of microsatellite chimerism (e.g. see the 153-bp allele of Pinot Meunier in Fig. 2d).

The stability of an established chimeric state, demonstrated here, within a shoot meristem suggests that non-chimeric mutants arise from periclinal chimeras at a low frequency. The Pinot Meunier periclinal phenotype has existed and remained stable for hundreds of years. Thus, in most cases mutated microsatellite loci would give a unique 3-allele genotype, different to the original cultivar genotype. However, if a mutant cell line arose in one cell layer of the apex, and through propagation eventually became established in both cell layers, the possibility exists for such a non-chimeric clone to possess a new microsatellite DNA profile and be classified as a different variety if information on its origin is lacking. Regenerated L2 plants, while phenotypically similar to Pinot noir, were found to have a different microsatellite genotype showing that it is possible for two phenotypically similar plants of the same cultivar to have a different DNA profile. This has also been recently shown for clones of Riesling (Regner et al. 2000b). Caution should therefore be exercised when grapevine cultivar-identification decisions are based solely on a single microsatellite allele difference without investigating the origin of the plant or its phenotype.

Implications of chimerism for grapevine improvement

The apparent stability of the periclinal state of chimeric cultivars and clones of grapevine through vegetative propagation (Thompson and Olmo 1963) and the reasonable frequency of reports of natural chimeric sports (Thompson and Olmo 1963; Rives 1970) can be extrapolated to suggest that periclinal chimeras occur at an appreciable rate amongst many grapevine cultivars. The clonal differences observed during clonal selection for grapevine cultivar improvement may, in some cases, be due to a chimeric state and chimerism should be considered as a potential cause of clonal differences along with disease load, epigenetic and genetic differences.

Although it would be very difficult to accurately estimate the microsatellite loci mutation rate in grapevine somatic cells some general comments are warranted. The establishment of a periclinal chimera of grapevine is the result of a combination of a series of small probabilities. A somatic mutation must first occur in a cell which is, or will become, part of a shoot meristem and then be amplified to the point of domination of a distinct cell layer of that meristem. However it is formed, a periclinal shoot must then survive annual pruning and be selected for propagation.

Continuous vegetative propagation has probably extended the natural lifetime of many grapevine genotypes, and the different periods over which the various varieties have been cultivated could explain why some cultivars have produced more clonal variants than others. An alternative explanation is that some ancient cultivars like Pinot may be more prone to accumulate mutations; for example, studies in other eukaryotes have found that defects in mismatch repair lead to a higher incidence of mutations including microsatellite mutations (e.g. Strand et al. 1993; Karran and Bignami 1994). The two microsatellite mutations identified in Pinot Meunier are of interest in this respect.

The use of somatic embryogenesis to obtain pure mutations from periclinal chimeric plants has both advantages and disadvantages for grapevine improvement. The contribution to grapevine improvement which could be made by releasing potential intraclonal variability from the two cell layers of chimeras has been discussed (Thompson and Olmo 1963). If superior clonal selections are used, the recovery of non-chimeric mutants which consist entirely of cells with either the L1 or L2 genotypes may generate new agronomically useful phenotypes. In addition, beneficial mutations which arise in the L1 layer would be made available for breeding purposes after plant regeneration from L1 embryogenic tissue.

Separation of chimeric cell layers of established grapevine cultivars may also facilitate gene isolation by identification of gene expression differences because both cell lines involved in a periclinal chimera can be regarded as near-isogenic. Grapevine near-isogenic lines have never been obtained through breeding due to severe inbreeding depression. This strategy could be applied to investigate the genetic differences between the Pinot Meunier L1 dwarf mutant and the L2 genotype, for example.

For grapevine improvement, transgenic systems appear to have an advantage over conventional breeding because modifications can be made directly to the clonally propagated traditional cultivars upon which the viticultural industries have been established. This is possible if, apart from the introduced DNA, the genome of transgenic plants is unchanged from that of the original cultivar. Attempts to regenerate grapevine transgenics via organogenesis have been unsuccessful (Baribault et al. 1990; Colby et al. 1991) but this impasse has been overcome using regeneration via embryogenesis (Mauro et al. 1995; Perl et al. 1996; Scorza et al. 1996; Franks et al. 1998). Results presented here show that it will not be possible to recover true-to-type transformed plants from cultivars and their clones which are chimeras since transgenic plants will differ from the original parent by more than just the introduced gene. Our results with Pinot Meunier graphically illustrate this; however, other cases involving chimeric cultivars may not be so easily detected and involve more subtle changes which affect fruit quality and plant performance. The fruit is of special interest with regard to L1, L2 genetic differences as the berry skin is a primary source of colour and some flavour compounds, with the internal berry flesh contributing mostly sugar and acid.

This demonstration of microsatellite chimerism in grapevines suggests further research is required into its relevance to DNA profiling studies, as well as into the cause and extent of genetic variability between clones of a cultivar. New approaches to intra-cultivar genetic improvement may be possible with the separation of genetically distinct L1- and L2- derived plants regenerated from embryogenic cultures of superior clones. The possibility exists that grapevine plants with or without transgenes which originate from somatic embryogenesis will require new clonal, and perhaps cultivar, identities if their DNA profile or agronomic performance is significantly different from the original cultivar clone.

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