MOLECULAR PROFILING OF *VITIS VINIFERA* CHARDONNAY OBTAINED BY SOMATIC EMBRYOGENESIS

PROFIL MOLÉCULAIRE DE *VITIS VINIFERA* CHARDONNAY OBTENU PAR EMBRYOGENESE SOMATIQUE

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Abstract : With the help of microsatellite profiling, we showed that *Vitis vinifera* Chardonnay clone 96 is a periclinal chimera plant which is composed at least of two distinct cell layers. Performing somatic embryogenesis allowed us to separate the two cell layers and to regenerate L1 plants. These regenerated L1 plants did not show phenotypic differences to the parental clone when grown in greenhouse conditions, suggesting therefore that the phenotype of Chardonnay 96 did not result of an interaction between the two distinct cell layers L1 and L2.

Résumé : L'analyse de microsatellites montre que *Vitis vinifera* Chardonnay clone 96 est une plante chimérique composée au moins de deux couches cellulaires distinctes. L'obtention de somaclones de Chardonnay 96 par l'embryogenèse continue ou embryogenèse somatique nous a permis de séparer les deux couches cellulaires et de régénérer des plantes constituées exclusivement de cellules issues de la couche L1. Cultivées en serre, ces plantes régénérées L1 ne montrent pas de différence phénotypique par rapport au clone parental. Nos résultats suggèrent donc que le phénotype du Chardonnay 96 ne résulte pas de l'interaction des deux couches cellulaires distinctes L1 et L2.

Key words : grapevine, Chardonnay, chimerism, somatic embryogenesis, L1

Mots clés : vigne, Chardonnay, chimère, embryogénèse continue, L1

INTRODUCTION

The grapevine meristem is considered to be composed at least of two distinct cell layers (L1 and L2) (THOMPSON and OLMO, 1963), which can produce a chimeric tissue structure. For example, *Vitis vinifera* cv. Pinot Meunier phenotype is due to the interaction of genetically distinct cell layers (FRANKS *et al.*, 2002). When the cell layers of Pinot Meunier periclinal chimera were separated by passage through somatic embryogenesis, regenerated plants showed distinct DNA profiles which proved to be different from that of the parent plant. Regenerated somaclones also showed a novel phenotype. These observations illustrated that transformed grapevines, regenerated via embryogenesis, could differ from the original parent in the introduced gene and the possible non-chimeric profile. As a consequence, transformation and regeneration via embryogenesis with putative chimeric cultivars would need genotypic and phenotypic analyses.

Microsatellites are tandem repeats of short (1-6 bp) units flanked by very conserved stretches. They are frequently found and rather evenly spaced throughout eukaryotic genomes (GOODFELLOW, 1992; POWELL *et al.*, 1996). Microsatellite-based markers are mono-locus, multiallelic, inherited as a co-dominant Mendelian trait, and easy to analyse. For all these characteristics they have become favoured markers to identify plants and animals. Microsatellite markers of the grapevine genome have been largely developed. More than three hundreds are now available and widely as well as very efficiently used for identification of cultivars (for review, see SEFC *et al.*, 2001).

Recently RIAZ *et al.* (2002) used microsatellite markers for the detection of reproducible intra-cultivar polymorphism in *Vitis vinifera* Chardonnay and Pinot noir. Furthermore, some of these microsatellite markers made it possible to differentiate the two cell layers L1 and L2. In the present work, we used microsatellite markers to characterise the L1 and L2 layers in leaves, rootlets and wood tissues of Chardonnay clone 96. We also describe the regeneration via embryogenesis for this cultivar and the separation of the genotypically distinct cell layers thanks to somatic embryogenesis.

MATERIALS AND METHODS

I - PLANT MATERIAL

Vitis vinifera cv. Chardonnay clone 96, was obtained from ENTAV (Établissement National Technique pour l'Amélioration de la Viticulture, Le Grau du Roi, France), the national repository for registered grape clones in France. Forced adult plants were maintained in a growth chamber at 25 ± 0.5°C, 70 ± 10 % RH with a 16 h - photoperiod of 50 µE.m^{-2}.s^{-1}.

II - INITIATION OF PRIMARY EMBRYOGENIC CALLUS

Anthers were dissected and grown as described by MAURO *et al.* (1986). For long-term culture of embryogenic callus, subcultures were performed every three weeks on the MPM medium described by PERRIN *et al.* (2001). All the cultures were maintained at 25 ± 0.5 °C, 70 ± 10 % RH with a 16 h - photoperiod of 50 µE.m^{-2}.s^{-1}, except the callus initiation which was performed in the dark.

III - INITIATION OF SECONDARY EMBRYOGENIC CALLUS

Embryos from primary embryogenic callus were cut off and transferred into Petri dishes containing half-strength Murashige and Skoog medium (MS medium) (MURASHIGE and SKOOG, 1962) supplemented with 20 g.l^{-1} sucrose, 0.7 % agar, 2.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 µM 6-benzylaminopurine (BA; N6-benzyladenine). Incubation was done at 25 ± 0.5°C in the dark, during 3 weeks. Then calli were subcultured every three weeks on MPM medium and maintained under the same conditions as previously described.

IV - REGENERATION AND ACCLIMATIZATION OF SOMACLONES

Embryos were carefully excised and transferred into Petri dishes with half-strength MS medium containing 20 g.l^{-1} sucrose, 0.7 % agar and 0.4 µM BA. After two weeks at 25 ± 0.5 °C and 16 h light, the growing embryos were individually transferred into tubes containing half-strength MS medium. Plantlets with rootlets were transferred to soil and allowed to acclimatize in a growth chamber for about three weeks before transfer to a greenhouse.

V - DNA EXTRACTION

As a control, leaves were harvested from *in vitro* - and greenhouse-grown somaclones and also from Chardonnay 96 greenhouse-grown cuttings as a control. Rootlets were taken from *in vitro*-grown plants. For the wood, some bark was removed from hardwood cuttings obtained from ENTAV. About 80 mg of tissue was ground in liquid nitrogen, the total DNA was extracted with the Qiagen DNeasy Plant mini-kit (Qiagen, Hilden, Germany) as described by the supplier.

VI - AMPLIFICATION OF MICROSATELLITES AND POLYMORPHISM DETECTION

The DNA was analyzed using two pairs of primers flanking two different microsatellite regions: VMC 6c10 and VMC 5g7 (Vitis Microsatellites Consortium, Dr. Rosa Arroyo Garcia and Dr. Kirsten Wolff), respectively marked with the fluorophores HEX and FAM. PCR amplification was performed with a programmable thermal cycler (Applied Biosystem, GeneAmp PCD system 2700). The amplification products were separated by capillary electrophoresis and detected with an ABI PRISM 310 Genetic Analyser (Applied Biosystem).

RESULTS

I - GENOTYPIC ANALYSIS OF LEAVES, ROOTLETS AND WOOD TISSUES OF CHARDONNAY 96

The three alleles described by RIAZ *et al.* (2002) were detected in leaves with VMC 6c10 : the standard 114 bp and 140 bp alleles and an additional 142 bp variant allele (table I). The standard alleles were defined by RIAZ *et al.* as the most frequently detected alleles in different clones of the same cultivar. VMC 5g7 revealed in leaves the two standard 198 bp and 220 bp alleles and a 222 bp variant allele. The same results were observed with leaf extracted DNA from *in vitro* or greenhouse-grown plants. In the DNA extracted from rootlets and wood tissues, only the two pairs of alleles 114-142 and 198-220 were detected with VMC 6c10 and VMC 5g7 respectively (table IA).

II - INDUCTION OF SOMATIC EMBRYOGENESIS AND GROWTH OF SOMACLONES

Somatic embryogenesis was obtained from anther cultures. Primary somatic embryos were obtained from anther-derived embryogenic calli after a 2 months period. Secondary embryos were obtained 1 month after initiation of embryogenic calli from primary embryos. Efficient plant growth was further obtained with a number of somaclones. The plants, respectively grown in the growth chamber and the greenhouse, did not show an atypical phenotype in comparison with vegetatively propagated Chardonnay 96. In some cases, juvenile characteristics such as asymetric leaves could be observed.

Table I - VMC 5g7 and VMC6c10 locus alleles of A) vegetatively propagated Chardonnay 96
B) plants regenerated from primary or secondary somatic embryos of Chardonnay 96

Allèles VMC 5g7 et VMC6c10 de A) Chardonnay 96
B) plantes régénérées par embryogenèse somatique primaire et secondaire à partir d'embryons de Chardonnay

A

plant	VMC 6c10 locus allele (bp)	VMC 5g7 locus allele (bp)	layer
Standard genotype (Riaz *et al.* ,2002)	**114 : 140**	**198 : 220**	
Chardonnay 96 leaf	114 : 140 : 142	198 : 220 : 222	L1; L2
Chardonnay 96 wood tissue	114 : : 142	198 : 220	L2
Chardonnay 96 rootlet	114 : : 142	198 : 220	L2

B

plants	VMC 6c10 locus allele (bp)	VMC 5g7 locus allele (bp)	initial layer
18 primary somaclones			
in vitro leaf	114 : 140	198 : 222	
greenhouse leaf	114 : 140	198 : 222	L1
rootlet	114 : 140	198 : 222	
6 secondary somaclones			
in vitro leaf	114 : 140	198 : 222	
greenhouse leaf	114 : 140	198 : 222	L1
primary somaclone N° 21			
in vitro leaf	114 : 140 : 142	198 : 222	
greenhouse leaf	114 : 140 : 142	198 : 222	L1
rootlet	114 : : 142	198 : 222	

III - GENOTYPIC ANALYSIS OF CHARDONNAY 96 SOMACLONES

Eighteen primary somaclones and 6 secondary somaclones were analysed. In leaves from *in vitro* and greenhouse-grown plants, the standard genotype (114, 140) was visualised with VMC 6c10 (table IB). Interestingly in leaves of one primary somaclone (N° 21) a variant 142 bp allele was also detected in addition to the standard alleles displaying the typical profile of vegetatively propagated Chardonnay 96 leaves. For VMC 5g7 in addition to the standard 198 bp allele, the 222 bp variant allele was detected in leaves from all somaclones including N° 21 whereas 220 bp allele was absent (table IB). In the rootlets from all somaclones, except N° 21, the standard genotype was visualised with VMC 5g7 and with VMC 6c10. In the rootlets of somaclone N° 21, two alleles were detected with VMC 6c10 : the standard 114 bp allele and the 142 bp variant allele. With VMC 5g7 the 198 bp standard allele and the 222 bp variant allele were detected.

DISCUSSION

Our results showed that Chardonnay clone 96 is a chimeric plant which is composed at least of two distinct layers L1 and L2. In grapevine, apical meristems are composed of two or more layers forming the tunica in addition to a corpus (PRATT, 1959; THOMPSON and OLMO, 1963; MORRISON, 1991). The outer layer L1 is stable for the mutations and histogenetically distinct from the other layers, but the inner tunica layer L2 undergoes occasional periclinal cell divisions, which lead to layer mixing.

In the leaf tissue from Chardonnay 96, which is derived from both the outer tunica layer L1 and the inner cell layer L2, a microsatellite marker (VMC 5g7) revealed the two standard alleles previously defined by RIAZ *et al.* (2002) and a variant allele. Wood tissues and roots, which originated exclusively from the L2 layer (PRATT, 1959), presented only the two standard alleles. The presence of a third allele in leaf suggested that Chardonnay could be a periclinal chimera in which a mutant allele was present only in the L1 layer, as described by RIAZ *et al.* (2002). For the second microsatellite marker (VMC 6c10), a third allele was detected in leaf, wood tissue and roots. This mutant allele replaced one of the standard alleles in woods and roots, but in leaves the new allele was present simultaneously with the two standard alleles. These results suggest that the 2 bp-mutation resulting in the replacement of the 140 bp allele by the 142 bp allele only occurred in L2. These results contradict those reported by RIAZ *et al.* (2002) who reported the absence of the 140 bp allele in root- and wood-tissues as well as in leaves. This could only be the case if the mutated

cells had diffused and multiplied both in L1 and L2 layers. The results reported by RIAZ *et al.* were based on visual interpretation of electrophoretic profiles of amplification products. Such observations could be biased by the DNA polymerase slippage. Our analyses with an ABI PRISM allowed to differentiate between an allele and the different stutter bands, without ambiguity.

Embryogenesis might generate new grapevine phenotypes. For example, the separation of chimeric cell layers of Pinot Meunier through somatic embryogenesis generated plants that had distinct DNA profiles and had novel phenotypes which were different from those of the parent plant (FRANKS *et al.*, 2002). In the present work involving the chimeric cultivar Chardonnay 96, all 25 somaclones deriving from L1 cells were shown to display a DNA profile distinct from that of the parent plants, though these clones never showed atypical phenotypes compared to vegetatively propagated Chardonnay 96. Two hypotheses could be proposed: first, phenotypic differences of our L1 somaclones might involve more subtle changes which would affect fruit quality or plant performance for instance, and had not yet been detected. In the second hypothesis, the L2 genotype would not significantly participate in the phenotypic expression in the L1-L2 chimera.

Anthers had been the most widely used organs for grapevine somatic embryogenesis initiation. The somatic embryos of *Vitis* seemed to develop from single cells (KRUL and WORLEY 1977; FAURE *et al.*, 1996). Furthermore both L1 and L2 cell layers of the anther filament seemed to be competent to form embryogenic calli (FRANKS *et al.*, 2002). Regeneration of somaclones from chimeric cultivars could give rise to non-chimeric plants entirely composed of either L1 or L2 cells.

In our conditions only L1 cells developed into embryos. Analysis of embryogenic calli (data not shown) suggested that they were composed exclusively of L1 cells and that L2 cells could therefore not multiply into callus. This hypothesis is in accordance with the observation of the phenotypic similarity between our somaclones and vegetatively propagated Chardonnay 96. Though Chardonnay 96 is a chimera, its phenotype may not be the result of an interaction between the genetically different L1 and L2 layers, but L2 could be a silenced layer for the phenotypic expression of Chardonnay 96. If this was true, regeneration via embryogenesis for Chardonnay 96 could result in an unchanged phenotype and would be well suited for gene transfer.

In leaves of somaclone N° 21, the typical profile of vegetatively propagated Chardonnay 96 leaves (L1 + L2) was detected with VMC 6c10. In contrast, the typical L1 profile was shown with VMC 5g7. Moreover, the rootlet genotypic profile was a L2 profile for VMC 6c10 and a L1 profile for VMC 5g7. These results may suggest that a mutation occurred in the initial cell from which root tissues developed but not in the initial cell from which external tissues developed. This mutation led to a 142 bp allele already detected in Chardonnay 96 mother plant, which was considered as a homoplasy. This hypothesis would be in accordance with the fact that the most frequently observed allelic size variation is the addition of one motif (HOCQUIGNY, 2003).

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