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Somatic embryogenesis from anthers of the autochthonous *Vitis vinifera* cv. Domina leads to *Arabis mosaic virus*-free plants

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Abstract Attempts to conserve and utilise autochthonous grapevine germplasm in modern breeding programmes, are sometimes faced with the challenge that virus-free plants of old grapevine varieties and clones are hard to find. From 50 year-old vineyards in Frankonia the *Vitis vinifera* cv. Domina was selected showing particularly interesting loose-bunch architecture with fewer berries. However this valuable germplasm was carrying an *Arabis mosaic virus* (ArMV) infection requiring a reliable and effective method to produce healthy mother plants for clonal selection. Somatic embryogenesis was established from anthers as the most promising technical approach. The absence of ArMV in 46 regenerated plant lines was confirmed by ELISA and IC-RT PCR, repeated after different time intervals *in vitro* and *in vivo* after acclimatisation, and after one dormancy period under glasshouse conditions. Morphologically, all grapevines appeared true-to-type, and a screening of 20 plants by flow cytometry to determine the ploidy level and to exclude the risk of undesired genetic variability confirmed that all tested plants were diploid. Field evaluations of the initially selected bunch traits are currently underway.

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Viticulture of today is based on the cultivation of clones selected for stable and high yield, high quality and other beneficial features. Planting clonal material provides many advantages, but on the other hand leads to a loss of genetic variability within a cultivar and deprives breeders of valuable sources for new and different clones and also special traits for cross-breeding (Bleser et al. 2004). In many European programmes for grapevine growing regions have been scrutinised for vineyards planted more than 50 years ago, and harbouring ancient indigenous cultivars or old clones of current cultivars. This recent raise in interest in conservation and utilisation of autochthonous germplasm (Rühl et al. 2006) documented also in the European *Vitis* database (http://www.genres.de/eccdb/vitis/), reflects a novel approach to germplasm management and utilisation.

In areas with long-term viticulture, the phytosanitary status of old vines is critical. Particularly important in this respect are virus diseases, such as leaf roll and fanleaf diseases, spread by mealybugs and nematodes respectively, because they are responsible for great economic losses. Grapevine fanleaf disease is caused by a complex of nepoviruses, distinguishable by a specific connection to their nematode vector species. ArMV, involved in the etiology of fanleaf disease of grapevines in central

European countries, occurs worldwide, but interestingly has a major economic impact in viticulture only in cooler climates, where its vector, *Xiphinema diversicaudatum*, is present (Trudgill et al. 1983). Many infections are latent or symptoms can vary greatly depending on cultivar, rootstock and environmental conditions. Fanleaf disease can have devastating effects causing crop losses of up to 50% due to reduced growth, dieback and severe dropping of fruit (Bovey et al. 1980).

Prevention of virus spread in the vineyard is important since once a viral disease is established in the field there is no treatment or cure. Therefore efforts to produce planting material free of *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus-1*, *-2* and *-3*, ArMV and *Grapevine virus A* are considered important control measures (Gambino et al. 2008).

Several authors (Goussard and Wiid 1992; Schaefers et al. 1994; Gambino et al. 2006, 2008; Popescu et al. 2003) have obtained plants cured from GFLV, *Grapevine leafroll-associated viruses*, or *Grapevine fleck virus* through somatic embryogenesis, alone or in combination with thermotherapy, but the method proved highly genotype-dependent, and the effectiveness varied according to the source of embryos and virus strains (Torres-Vinals et al. 2004). Somatic embryogenesis also represents a valuable technique for the production and storage of pathogen-free plant material (Das et al. 2006).

This study was undertaken with the aim of revitalising a collection of grapevines from old vineyards in Frankonia, including Domina, a German red wine cultivar. This cultivar results from a cross of Blauer Portugieser and Blue Pinot Noir (Schartl and Engelhart 2005) and is grown on about 600 ha in Germany. Although selected plants did not show visible symptoms, all of them tested positive in ELISA for different grapevine viruses, e.g. GFLV, GLRaVs, ArMV, some even carrying multiple infections. A vine with a single ArMV infection was chosen to establish new healthy clones.

Shoots from the selected clone were cut at dormancy, treated with Chinosol and propagated as two-bud cuttings in the greenhouse until flowers set. Inflorescences were collected approximately two weeks before anthesis at the appropriate developmental stage of anthers (Gribaudo et al. 2004), the late uninucleate stage of pollen grains, as confirmed by karyotyping. For callus initiation three media were tested: PIV (Franks et al. 1998), NN/G+ (Gölles et al. 2000) and R6E (da Câmara Machado et al. 1995). Embryogenic calli were cultured on R6E and on NB2 (Gölles et al. 2000). Somatic embryos were transferred to hormone-free SH (Schenk and Hildebrandt 1972) or MS (Murashige and Skoog 1962) for germination. For shoot elongation and root induction the rooting medium (RM) 15B (Gölles et al. 2000) was used.

Cultures were maintained in the dark at 26°C, and subcultured at monthly intervals. Germination and regeneration experiments were carried out under light (16 h light, 8 h darkness) at 26°C and successfully propagated lines were acclimatised to greenhouse conditions.

Anthers taken into culture initiated callus formation in about six weeks. After three months in culture anthers formed calli with different efficiency depending on the culture media: 3% on PIV (12/407), 4% on NN/G+ (18/452) and 25% on R6E (100/399). Calli also differed in morphological appearance, in colour and texture, e.g. on PIV calli were brown and partially red, on NN/G+ transparent, brown and greenish, on R6E mainly brown. Anthers cultured on PIV directly entered somatic embryogenesis. Responding anthers were transferred to R6E medium for a further three months, and subsequently to NB2 medium. Green embryos or shoot-like structures were transferred to RM for elongation and rooting (Sommerbauer 2004). Well developed embryos used for germination and regeneration experiments required two months to develop into plantlets and finally 46 lines were acclimatised to greenhouse conditions.

Leaves of 46 micropropagated Domina plantlets derived from single embryos, were tested repeatedly for the absence of ArMV by DAS-ELISA using a commercial kit according to the manufacturer's recommendations (BIOREBA, Switzerland). Healthy and ArMV-infected grapevine leaves from the Vienna Collection of Fruit Tree Pathogens (http://www.boku.ac.at/iam/pbiotech/phytopath/col.html) were used as negative or positive controls, respectively. Optical densities were measured at 405/492 nm after 30–120 min. Samples were regarded as positive if their $OD_{405\ nm}$ value was at least twice the average reading of the healthy control.

Absence of ArMV was further confirmed after acclimatisation to greenhouse conditions by immunocapture-reverse transcription polymerase chain

reaction (IC-RT PCR). Plant extracts were processed in 0.5 mL tubes coated with 50 µL of anti-ArMV-IgG (BIOREBA, Switzerland) according to the manufacturer's recommendations for DAS-ELISA to detect ArMV. The viral RNA, released from the antibody-bound virus particles with 10 µL of Triton X100 0.1% (10 min, 65°C), was used directly in a RT PCR. The primer pair ArMV 31-fw (5′-CCTAAAGATTGCCAGGCGGG-3′) and ArMv 1313-rev (5′-CGAGATGCTCCATCCATGCC-3′) was used to amplify a fragment (782 bp) in the ArMV CP gene (Accession Number X55460.1). PCR-cycling conditions consisted of an initial step at 42°C for 45 min followed by 95°C for 15 min and 35 cycles of 60 s at 95°C, 60 s at the annealing temperature 60°C and 2 min at 72°C. A final step of 10 min at 72°C ended the cycle. Extracts of healthy and ArMV-infected grapevine leaves from the Vienna Collection of Fruit Tree Pathogens (http://www.boku.ac.at/iam/pbiotech/phytopath/col.html) were used as controls.

In this study 46 plantlets, derived through somatic embryogenesis from anthers of *V. vinifera* Domina were analysed 1, 4 and 32 months after acclimatisation by ELISA and IC-RT PCR, indicating a 100% elimination rate of ArMV, as recently also achieved successfully for GLFV, another nepovirus, by Gambino et al. (2008). However earlier reports had mentioned that somatic embryogenesis alone was not effective in eradicating GFLV (Goussard and Wiid 1992). This method has also been successfully used to eliminate *Citrus psorosis virus* from three citrus species for the production of healthy citrus stocks (D'Onghia et al. 2001). Alternatively, the use of somatic embryogenesis combined with heat shock treatments was shown to be effective as a method to eliminate viruses from grapevines (Goussard and Wiid 1992).

All plants acclimatised to greenhouse conditions appeared true-to-type and no evidence of somaclonal variation could be observed. The occurrence of genetic variation after somatic embryogenesis from anthers has been reported in some cases (Popescu et al. 2002), whereas other authors (Martinelli et al. 2004) could not detect genetic differences between the donor plants and the cells involved in somatic embryogenesis by SSR-analysis.

To further confirm trueness to type, the ploidy level of regenerated plants was checked by flow cytometry (CyFlow® ML, software Partec FloMAX, Partec GmbH, Germany) according to Baranyi and Greilhuber (1996), modified by Temsch (2003), because conventional cytogenetic techniques, e.g. karyotyping, would be laborious, time-consuming, and not always conclusive, given the high number and small size of chromosomes ($2n=38$) of *V. vinifera*. Although it could be anticipated that diploid as well as haploid regenerants could arise from anther explants (Salunkhe et al. 1999), all 20 anther-derived plants tested presented mean 1C values of 0.60 and were assigned to a diploid level. No haploid or aneuploid plants were observed, agreeing with the findings of Lima et al. (2003) on anther-derived somatic embryos in *V. vinifera* cv. Periquita, and further confirms the observations of Rajasekaran and Mullins (1983), that haploid cells from anthers do not contribute to the formation of somatic embryos.

Acclimatised plants have been planted in an experimental vineyard at the Bavarian State Institute to compare the material obtained after virus elimination to the original Domina clones. Particular attention will focus on the characteristic traits for which the plants were selected, i.e. loose bunches and fewer berries per bunch, or whether these traits were correlated with the initial virus infection.

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